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=> s l1 and epidermal growth factor receptor

L2 7773 L1 AND EPIDERMAL GROWTH FACTOR RECEPTOR

=> s l2 and EGFR

L3 2816 L2 AND EGFR

=> s l3 and inhibits tyrosine phosphorylation

L4 0 L3 AND INHIBITS TYROSINE PHOSPHORYLATION

=> s l3 and tyrosine phosphorylation

L5 217 L3 AND TYROSINE PHOSPHORYLATION

=> s l5 and block

L6 46 L5 AND BLOCK

=> s l6 and internalized

L7 5 L6 AND INTERNALIZED

=> s l7 and degradation

L8 0 L7 AND DEGRADATION

=> dup remove l7

PROCESSING COMPLETED FOR L7
L9 1 DUP REMOVE L7 (4 DUPLICATES REMOVED)

=> d l9 cbib abs

L9 ANSWER 1 OF 1 MEDLINE DUPLICATE 1
93179513 Document Number: 93179513. PubMed ID: 8382707. Anti-**epidermal growth factor receptor** monoclonal **antibodies** affecting signal transduction. Reins H A; Steinhilber G; Freiberg B; Anderer F A. (Friedrich-Miescher-Laboratorium der Max-Planck-Gesellschaft, Tuebingen, Federal Republic of Germany.) JOURNAL OF CELLULAR BIOCHEMISTRY, (1993 Feb) 51 (2) 236-48. Journal code: HNF; 8205768. ISSN: 0730-2312. Pub. country: United States. Language: English.

AB Monoclonal **antibodies** prepared against tyrosine phosphorylated **epidermal growth factor receptor** (**EGFR**) were tested for their effects on transmembrane signal transduction in A431 tumor cells. Monoclonal **antibodies** (mab) defined by SDS-sensitive epitopes, i.e., epitopes with conformational specificity, were most effective. Mab 5-125 reacting with a site of the extracellular **EGFR** domain blocked EGF-binding and cell proliferation in vitro, as well as tumor growth in vivo. However, this mab appeared not to be **internalized** upon binding to **EGFR** and did not trigger **EGFR** autophosphorylation. In contrast, mab 5-D43, also defined by an SDS-sensitive epitope and reacting with an extracellular **EGFR** site, did not **block** EGF binding but was readily **internalized** after binding to **EGFR** of untreated A431 cells. This mab induced **EGFR tyrosine phosphorylation** in cell lysates and tyrosine-specific autophosphorylation of insolubilized **EGFR** immune complexes. Cell growth in vitro was greatly stimulated in the presence of mab 5-D43. Since interaction of mab 5-D43 with **EGFR** induced most EGF-specific functions, although it did not bind to the EGF-specific site of **EGFR**, we have to assume that binding of mab 5-D43 to **EGFR** induced a conformational shift that activated the cytoplasmic **EGFR** kinase site. On the other hand, activation and/or accessibility of the **EGFR** kinase site could be blocked by mab 1-594, which is defined by an SDS-insensitive protein epitope of the cytoplasmic **EGFR** domain. Blocking of the **EGFR** kinase site by mab 1-594 also abolished EGF-induced **tyrosine phosphorylation** of endogenous cellular substrates with molecular masses of 145, 97, 85, 37, and 32 kDa, as well as of exogenous substrates such as GAT copolymer.

=> dup remove l6

PROCESSING COMPLETED FOR L6
L10 13 DUP REMOVE L6 (33 DUPLICATES REMOVED)

=> s l10 and 63 KD

L11 0 L10 AND 63 KD

=> d his

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L1      2245934 S ANTIBOD?
L2         7773 S L1 AND EPIDERMAL GROWTH FACTOR RECEPTOR
L3         2816 S L2 AND EGFR
L4            0 S L3 AND INHIBITS TYROSINE PHOSPHORYLATION
L5          217 S L3 AND TYROSINE PHOSPHORYLATION
L6           46 S L5 AND BLOCK
L7            5 S L6 AND INTERNALIZED
L8            0 S L7 AND DEGRADATION
L9            1 DUP REMOVE L7 (4 DUPLICATES REMOVED)
L10          13 DUP REMOVE L6 (33 DUPLICATES REMOVED)
L11           0 S L10 AND 63 KD
```

=> d l10 1-13 cbib abs

L10 ANSWER 1 OF 13 MEDLINE DUPLICATE 1
2001504480 Document Number: 21152854. PubMed ID: 11255078. Development of
ABX-EGF, a fully human anti-EGF receptor monoclonal **antibody**, for cancer therapy. Yang X D; Jia X C; Corvalan J R; Wang P; Davis C G. (Abgenix, Inc., 7601 Dumbarton Circle, Fremont, CA 94555, USA.. yang_xd@abgenix.com) . CRITICAL REVIEWS IN ONCOLOGY/HEMATOLOGY, (2001 Apr) 38 (1) 17-23. Ref: 24. Journal code: AGO; 8916049. ISSN: 1040-8428. Pub. country: Ireland. Language: English.

AB Overexpression of **epidermal growth factor receptor** (**EGFr**) has been demonstrated on many human tumors, and the increase in receptor expression levels has been linked with a poor clinical prognosis. Blocking the interaction of **EGFr** and the growth factors could lead to the arrest of tumor growth and possibly result in tumor cell death. To this end, using XenoMouse technology, ABX-EGF, a human IgG2 monoclonal **antibody** (mAb) specific to human **EGFr**, has been generated. ABX-EGF binds **EGFr** with high affinity (5x10(-11) M), **blocks** the binding of both EGF and transforming growth factor-alpha (TGF-alpha) to various **EGFr**-expressing human carcinoma cell lines, and inhibits EGF-dependent tumor cell activation, including **EGFr** **tyrosine phosphorylation**, increased extracellular acidification rate, and cell proliferation. In vivo ABX-EGF prevents completely the formation of human epidermoid carcinoma A431 xenografts in athymic mice. More importantly, administration of ABX-EGF without concomitant chemotherapy results in complete eradication of established tumors. No tumor recurrence was observed for more than 8 months following the last **antibody** injection, further indicating complete tumor cell elimination by the **antibody**. Inhibition of human pancreatic, renal, breast and prostate tumor xenografts which express different levels of **EGFr** by ABX-EGF was also achieved. Tumor expressing more than 17000 **EGFr** molecules per cell showed significant growth inhibition when treated with ABX-EGF. ABX-EGF had no effect on **EGFr**-negative tumors. The potency of ABX-EGF in eradicating well-established tumors without concomitant chemotherapy indicates its potential as a monotherapeutic agent for treatment of multiple **EGFr**-expressing human solid tumors, including those where no effective chemotherapy is available. Utilization of mAbs directed to growth factor receptors as cancer therapeutics has been validated recently by the tumor responses obtained from clinical trials with Herceptin, the humanized anti-HER2 **antibody**, in patients with HER2 overexpressing metastatic breast cancer. Being a fully human

antibody, ABX-EGF is anticipated to exhibit a long serum half-life and minimal immunogenicity with repeated administration, even in immunocompetent patients. These results demonstrate the potent anti-tumor activity of ABX-EGF and its therapeutic potential for the treatment of multiple human solid tumors that overexpress **EGFr**.

L10 ANSWER 2 OF 13 MEDLINE DUPLICATE 2
2001078249 Document Number: 20545480. PubMed ID: 10982794. Stimulation of
the mitogen-activated protein kinase cascade and **tyrosine phosphorylation** of the **epidermal growth factor receptor** by hepatopoietin. Li Y; Li M; Xing G; Hu Z; Wang Q; Dong C; Wei H; Fan G; Chen J; Yang X; Zhao S; Chen H; Guan K; Wu C; Zhang C; He F. (Department of Genomics and Proteomics, Beijing Institute of Radiation Medicine, Chinese National Human Genome Center at Beijing, 27 Taiping Road, Beijing 100850, China.) JOURNAL OF BIOLOGICAL CHEMISTRY, (2000 Dec 1) 275 (48) 37443-7. Journal code: HIV. ISSN: 0021-9258. Pub. country: United States. Language: English.

AB Hepatopoietin (HPO) is a novel human hepatotrophic growth factor, which specifically stimulates proliferation of cultured primary hepatocytes in vitro and liver regeneration after liver partial hepatectomy in vivo. Recently, the identification of the mitogenic effect of HPO on hepatoma cell lines and the existence of HPO-specific receptors indicate that HPO acts via its specific cell surface receptor. However, the molecular mechanism of HPO action is not fully elucidated. In this report, we examined the signal transduction events induced by HPO in hepatoma cell line (HepG2). Our results demonstrated that HPO induces phosphorylation of
mitogen-activated protein kinase kinase and mitogen-activated protein kinase (MAPK) in a rapid and transient manner. HPO stimulates **tyrosine phosphorylation** of **epidermal growth factor receptor** (**EGFR**). Furthermore, we observed that both MAPK activation and the mitogenic effect of HPO on HepG2 cells were completely blocked by AG1478, a specific
inhibitor of **EGFR** tyrosine kinase activity. However, the effects of HPO were not antagonized by an **EGFR**-blocking **antibody**, mAb528, which **blocks** the interaction between epidermal growth factor and **EGFR**, indicating that stimulation of **tyrosine phosphorylation** of **EGFR** by HPO was not mediated by epidermal growth factor. In contrast, genistein, a general tyrosine kinase
inhibitor, significantly attenuated the **tyrosine phosphorylation** of **EGFR** in response to HPO. In conclusion, our results suggest that **tyrosine phosphorylation** of **EGFR** may play a critical role in MAPK activation and mitogenic stimulation by HPO.

L10 ANSWER 3 OF 13 MEDLINE DUPLICATE 3
1999194218 Document Number: 99194218. PubMed ID: 10096554. Eradication of
established tumors by a fully human monoclonal **antibody** to the **epidermal growth factor receptor** without concomitant chemotherapy. Yang X D; Jia X C; Corvalan J R; Wang P;
Davis C G; Jakobovits A. (Abgenix, Inc., Fremont, California 94555, USA.. yang_xd@abgenix.com) . CANCER RESEARCH, (1999 Mar 15) 59 (6) 1236-43. Journal code: CNF; 2984705R. ISSN: 0008-5472. Pub. country: United States.
Language: English.

AB A fully human IgG2kappa monoclonal **antibody** (MAb), E7.6.3, specific to the human epidermal growth factor (EGF) receptor (**EGFr**) was generated from human **antibody**-producing XenoMouse strains engineered to be deficient in mouse **antibody** production and to

contain the majority of the human **antibody** gene repertoire on megabase-sized fragments from the human heavy and kappa light chain loci. The E7.6.3 MAb exhibits high affinity (KD = 5 x 10(-11) M) to the receptor, **blocks** completely the binding of both EGF and transforming growth factor alpha (TGF-a) to various **EGFr** -expressing human carcinoma cell lines, and abolishes EGF-dependent cell activation, including **EGFr tyrosine phosphorylation**, increased extracellular acidification rate, and cell proliferation. The **antibody** (0.2 mg i.p. twice a week for 3 weeks) prevents completely the formation of human epidermoid carcinoma A431 xenografts in athymic mice. More importantly, the administration of E7.6.3 without concomitant chemotherapy results in complete eradication of established tumors as large as 1.2 cm3. Tumor eradication of A431 xenografts was achieved in nearly all of the mice treated with total E7.6.3 doses as low as 3 mg, administered over the course of 3 weeks, and a total dose of 0.6 mg led to tumor elimination in 65% of the mice. No tumor recurrence was observed for more than 8 months after the last **antibody** injection, which further indicated complete tumor cell elimination by the **antibody**. The potency of E7.6.3 in eradicating well-established tumors without concomitant chemotherapy indicates its potential as a monotherapeutic agent for the treatment of multiple **EGFr**-expressing human solid tumors, including those for which no effective chemotherapy is available. Being a fully human **antibody**, E7.6.3 is expected to exhibit minimal immunogenicity and a longer half-life as compared with mouse or mouse-derivatized MAbs, thus allowing repeated **antibody** administration, including in immunocompetent patients. These results suggest E7.6.3 as a good candidate for assessing the full therapeutic potential of anti-**EGFr** **antibody** in the therapy of multiple patient populations with **EGFr**-expressing solid tumors.

L10 ANSWER 4 OF 13 SCISEARCH COPYRIGHT 2001 ISI (R)

1999:192347 The Genuine Article (R) Number: 172CU. Transforming growth factor-alpha short-circuits downregulation of the **epidermal growth factor receptor**. Ouyang X M; Gulliford T; Huang G C; Epstein R J (Reprint). HAMMERSMITH HOSP, IMPERIAL COLL SCH MED, DEPT METAB MED, ROOM 5S1, COMMONWEALTH BLDG, DU CANE RD, LONDON W12 0NN, ENGLAND (Reprint); IMPERIAL COLL SCH MED, DEPT METAB MED, LONDON, ENGLAND; IMPERIAL COLL SCH MED, DEPT ONCOL, LONDON, ENGLAND. JOURNAL OF CELLULAR PHYSIOLOGY (APR 1999) Vol. 179, No. 1, pp. 52-57. Publisher: WILEY-LISS. DIV JOHN WILEY & SONS INC, 605 THIRD AVE, NEW YORK, NY 10158-0012. ISSN: 0021-9541. Pub. country: ENGLAND. Language: English. *ABSTRACT IS AVAILABLE IN THE ALL AND IALL FORMATS*

AB Transforming growth factor-alpha (TGF alpha) is an **epidermal growth factor receptor** (**EGFR**) ligand which is distinguished from EGF by its acid-labile structure and potent transforming function. We recently reported that TGF alpha induces less efficient **EGFR** heterodimerization and downregulation than does EGF (Gulliford et al,, 1997, Oncogene, 15:2219-2223). Here we use isoform-specific **EGFR** and ErbB2 **antibodies** to show that the duration of **EGFR** signalling induced by a single TGF alpha exposure is less than that induced by equimolar EGF. The protein trafficking inhibitor brefeldin A (BFA) reduces the duration of EGF signalling to an extent similar to that seen with TGF alpha alone; the effects of TGF alpha and BFA on **EGFR** degradation are opposite, however, with TGF alpha sparing **EGFR** from downregulation but BFA accelerating EGF-dependent receptor loss. This suggests that BFA **blocks EGFR** recycling and thus shortens EGF-dependent receptor signalling, whereas TGF alpha shortens receptor signalling and thus **blocks EGFR** downregulation. Consistent with this, repeated application of TGF alpha is accompanied by prolonged **EGFR** expression and signalling, whereas similar application of EFF causes

receptor downregulation and signal termination. These findings indicate that constitutive secretion of pH-labile TGF alpha may perpetuate **EGFR** signalling by permitting-early oligomer dissociation and dephosphorylation within acidic endosomes, thereby extinguishing a phosphotyrasine-based downregulation signal and creating an irreversible autocrine growth loop. J. Cell. Physiol. 179:52-57, 1999. (C) 1999 Wiley-Liss, Inc.

L10 ANSWER 5 OF 13 EMBASE COPYRIGHT 2001 ELSEVIER SCI. B.V.
1998174905 EMBASE [Tyrosine kinase: Implications in tumorigenesis and new avenues for cancer treatment]. TYROSINE KINASE: IMPLICATIONS EN PATHOLOGIE
TUMORALE ET PERSPECTIVES THERAPEUTIQUES. Peyrade F.; Taillan B.; Lebrun C.; Baron V.; Dujardin P.. F. Peyrade, Svc. d'Hematologie-Medecine Interne, Hopital l'Archet I, BP 3079, 06202 Nice cedex 03, France. Revue de Medecine Interne 19/5 (366-372) 1998.
Refs: 15.
ISSN: 0248-8663. CODEN: RMEIDE. Pub. Country: France. Language: French. Summary Language: English; French.
AB The tyrosine kinase family includes growth factor receptor and cytoplasmic
enzymes. It plays a key role in normal cell division and abnormal cell proliferation and differentiation. The most common tyrosine kinases are the epidermal-growth factor (**EGFR**) and platelet-derived growth factor (PDGF) receptors, and a chromosome Philadelphia product, the Bcr-abl oncogene. Many studies have attempted to correlate clinical evolution of tumors with tyrosine kinase expression. However, clinical application of these new prognostic factors has not yet been demonstrated.
More recently, **tyrosine- phosphorylation** inhibitors (tyrphostin) have been developed in phase I studies. Results that were obtained show some objective responses in patients with glioblastoma and polymetastatic cancer. Another approach to **block** tyrosine kinase expression is the use of monoclonal **antibodies**. Trials using such **antibodies** have shown interesting preliminary results.

L10 ANSWER 6 OF 13 MEDLINE DUPLICATE 4
1998330492 Document Number: 98330492. PubMed ID: 9664130. **EGFR** blockade by tyrosine kinase inhibitor or monoclonal **antibody** inhibits growth, directs terminal differentiation and induces apoptosis in
the human squamous cell carcinoma HN5. Modjtahedi H; Affleck K; Stubberfield C; Dean C. (The Institute of Cancer Research, McElwain Laboratories, Belmont, Sutton, Surrey, UK.) INTERNATIONAL JOURNAL OF ONCOLOGY, (1998 Aug) 13 (2) 335-42. Journal code: CX5; 9306042. ISSN: 1019-6439. Pub. country: Greece. Language: English.
AB Human squamous cell carcinomas frequently overexpress the **epidermal growth factor receptor** (**EGFR**) and this is often associated with poor prognosis in patients with these cancers. The high level of expression of the **EGFR** provides an important target for therapy and we and others have shown that
monoclonal **antibodies** (mAbs) which **block** the activation of the receptor by the EGF family of ligands inhibit the growth
of **EGFR** overexpressing tumours in vitro and induce the regression of established tumours grown as xenografts in athymic mice. Inhibitors of the tyrosine kinase associated with the **EGFR** have also been shown to **block** receptor activation and prevent tumour cell proliferation. Using the **EGFR**-overexpressing head and neck carcinoma cell line HN5, we have compared the biological consequences of treatment with an inhibitor of **EGFR** tyrosine kinase (PD153035) with anti-**EGFR** monoclonal **antibodies** (mAbs) ICR63 or ICR80. We found that both the anti-**EGFR** mAbs and the TK

inhibitor produce similar biological changes namely, they inhibit the EGF and TGFa-induced **tyrosine phosphorylation** of the receptor and the growth in culture of HN5 cells. At concentrations above 100 nM, the TK inhibitor prevented the growth in culture of HN5 cells completely with an IC50 of 40 nM. With the anti-**EGFR** mAbs, growth of HN5 cells was inhibited completely at concentrations above 4 nM with an IC50 of 1 nM. More importantly we found that, like the anti-**EGFR** mAbs, treatment with the TK inhibitor directs HN5 cells to undergo terminal differentiation as monitored by the expression of cytokeratin 10. In addition, our results indicate that the growth inhibitory effects of the anti-**EGFR** agents also lead to induction of apoptosis as determined by 7-amino actinomycin D staining (7-AAD). We conclude that **EGFR** blockade by anti-**EGFR** mAbs or TK inhibitor influences the growth in culture of **EGFR** overexpressing tumours by directing terminal differentiation and inducing apoptosis.

L10 ANSWER 7 OF 13 EMBASE COPYRIGHT 2001 ELSEVIER SCI. B.V.DUPLICATE 5
1998070428 EMBASE Anti-**EGFR** monoclonal **antibodies** which act as EGF, TGF.alpha., HB-EGF and BTC antagonists **block** the binding of epiregulin to **EGFR**-expressing tumours. Modjtahedi H.; Komurasaki T.; Toyoda H.; Dean C.. H. Modjtahedi, Institute of Cancer Research, McElwain Laboratories, Sutton, Surrey SM2 5NG, United Kingdom. helmout@icr.ac.uk. International Journal of Cancer 75/2 (310-316) 19 Jan 1998.
Refs: 24.
ISSN: 0020-7136. CODEN: IJCNAW. Pub. Country: United States. Language: English. Summary Language: English.

AB Epiregulin is the newest member of the epidermal growth factor (EGF) family of ligands that was isolated from conditioned medium of the murine fibroblast-derived tumour cell line NIH3T3/T7. Here, using a panel of anti- **EGFR** receptor (**EGFR**) monoclonal **antibodies** (MAbs) directed against 4 distinct epitopes on the external domain of the receptor, we have investigated the importance of the **EGFR** in transmitting the biological action of epiregulin. We found that MAb ICR9, which enhances the binding of EGF, TGF.alpha., HB-EGF and betacellulin to the **EGFR**, also increases the binding of 125I-epiregulin to a number of **EGFR**-expressing tumour cell lines, including EJ, SKBR3, SKOV3, MDA-MB46B and HN5. In addition, anti-**EGFR** MAbs ICR15, ICR16, ICR61, ICR62 and ICR80, which **block** the binding of 125I-EGF to the **EGFR**, inhibit the binding of 125I-epiregulin to these tumour cell lines. Like EGF, we found that both the epiregulin-induced growth inhibition of HN5 and MDA-MB468 cells and **tyrosine phosphorylation** of the 170 kDa **EGFR** on HN5 cells are reversed in the presence of anti-**EGFR** MAbs ICR62 and ICR80. Surprisingly and unlike 125I-EGF, radiolabelled epiregulin bound very poorly to human bladder carcinoma EJ cells and its binding to SKOV3 cells was not inhibited efficiently in the presence of blocking **antibodies**. We conclude that the **EGFR** plays an important role in transmitting the biological action of epiregulin and that these effects could be blocked in the presence of anti-**EGFR** MAbs. The low level of binding of epiregulin compared with EGF to EJ cells suggests that the **EGFR** may not be the primary receptor for epiregulin.

L10 ANSWER 8 OF 13 SCISEARCH COPYRIGHT 2001 ISI (R)
97:193770 The Genuine Article (R) Number: WK897. Role of **epidermal growth factor receptor** and STAT-3 activation in autonomous proliferation of SUM-102PT human breast cancer cells. Sartor C I; Dziubinski M L; Yu C L; Jove R; Ethier S P (Reprint). UNIV MICHIGAN, DEPT RADIAT ONCOL, DIV RADIAT & CANC BIOL, SCH MED, 1331 E ANN ST, ANN ARBOR, MI 48109 (Reprint); UNIV MICHIGAN, DEPT RADIAT ONCOL, DIV

RADIAT & CANC BIOL, SCH MED, ANN ARBOR, MI 48109; H LEE MOFFITT CANC CTR, TAMPA, FL 33612. CANCER RESEARCH (1 MAR 1997) Vol. 57, No. 5, pp. 978-987.

Publisher: AMER ASSOC CANCER RESEARCH. PUBLIC LEDGER BLDG, SUITE 816, 150 S. INDEPENDENCE MALL W., PHILADELPHIA, PA 19106. ISSN: 0008-5472. Pub. country: USA. Language: English.

ABSTRACT IS AVAILABLE IN THE ALL AND IALL FORMATS

AB This report describes the isolation and characterization of a new human breast cancer cell line, SUM-102PT, obtained from a minimally invasive human breast carcinoma, SUM-102PT cells have a near diploid karyotype, and early-passage cells had minor chromosomal abnormalities including a 5, 12 and a 6, 16 reciprocal translocation. The culls were isolated and have been continually cultured in three defined media, one of which contains exogenous epidermal growth factor (EGF). SUM-102PT cells have also been carried in an EGF-free medium supplemented with progesterone. All SUM-102PT cells require EGF receptor (**EGFR**) activation for continuous growth, because incubation of the cells with **EGFR** -neutralizing **antibodies** or with **EGFR** kinase inhibitors **blocks** growth of these cells, Southern analysis indicates that the **EGFR** gene is not amplified in these cells; however, these cells express high levels of **EGFR** mRNA. Thus, SUM-102PT is representative of a class of human breast cancers characterized by high level **EGFR** expression in the absence of gene amplification, SUM-102PT cells cultured in EGF-free, progesterone-containing medium express high levels of constitutively active **EGFR**. Conditioned medium from SUM-102PT cells contains an EGF-Like mitogen that binds to a heparin-agarose affinity matrix with high affinity, Northern analysis for various EGF family members indicates that SUM-102PT cells synthesize heparin binding (HB)-EGF mRNA. HB-EGF protein is detectable on the surface of these cells by immunohistochemistry, and SUM-102PT cells are killed by diphtheria toxin, which acts by binding to IIB-EGF. Furthermore, HB-EGF **antibodies** partially neutralize the mitogenic activity of the conditioned medium, Thus, **EGFR** activation in SUM-102PT cells is mediated, at Least in part, by autocrine/juxtacrine stimulation by HB-EGF. SUM-102PT cells also express constitutively active STAT-3 homodimers. Constitutively tyrosine-phosphorylated STAT-3 homodimers were also detected in another breast cancer cell line, MDA468, which has an **EGFR** amplification and also has constitutive **EGFR** activity. Thus, SUM-102PT is a new human breast cancer cell line that expresses activated **EGFR** as a result of an autocrine/juxtacrine interaction with HB-EGF which, in turn, results in activation of STAT-3.

L10 ANSWER 9 OF 13 MEDLINE DUPLICATE 6

1998012851 Document Number: 98012851. PubMed ID: 9348331. Modulation of the Kv1.3 potassium channel by receptor tyrosine kinases. Bowlby M R; Fadool D A; Holmes T C; Levitan I B. (Department of Biochemistry and Volen Center for Complex Systems, Brandeis University, Waltham, Massachusetts 02254, USA.) JOURNAL OF GENERAL PHYSIOLOGY, (1997 Nov) 110 (5) 601-10. Journal code: I8N; 2985110R. ISSN: 0022-1295. Pub. country: United States.

Language: English.

AB The voltage-dependent potassium channel, Kv1.3, is modulated by the **epidermal growth factor receptor** (**EGFr**) and the insulin receptor tyrosine kinases. When the **EGFr** and Kv1.3 are coexpressed in HEK 293 cells, acute treatment of the cells with EGF during a patch recording can suppress the Kv1.3 current within tens of minutes. This effect appears to be due to **tyrosine phosphorylation** of the channel, as it is blocked by treatment with the tyrosine kinase inhibitor erbstatin, or by

mutation of the tyrosine at channel amino acid position 479 to phenylalanine. Previous work has shown that there is a large increase in the **tyrosine phosphorylation** of Kv1.3 when it is coexpressed with the **EGFr**. Pretreatment of **EGFr** and Kv1.3 cotransfected cells with EGF before patch recording also results in a decrease in peak Kv1.3 current. Furthermore, pretreatment of cotransfected cells with an **antibody** to the **EGFr** ligand binding domain (alpha-**EGFr**), which **blocks** receptor dimerization and tyrosine kinase activation, **blocks** the **EGFr**-mediated suppression of Kv1.3 current. Insulin treatment during patch recording also causes an inhibition of Kv1.3 current after tens of minutes, while pretreatment for 18 h produces almost total suppression of current. In addition to depressing peak Kv1.3 current, EGF treatment produces a speeding of C-type inactivation, while pretreatment with the alpha-**EGFr** slows C-type inactivation. In contrast, insulin does not influence C-type inactivation kinetics. Mutational analysis indicates that the EGF-induced modulation of the inactivation rate occurs by a mechanism different from that of the EGF-induced decrease in peak current. Thus, receptor tyrosine kinases differentially modulate the current magnitude and kinetics of a voltage-dependent potassium channel.

L10 ANSWER 10 OF 13 MEDLINE DUPLICATE 7

97126968 Document Number: 97126968. PubMed ID: 8971836. Inhibition of **epidermal growth factor receptor** -associated tyrosine kinase **blocks** glioblastoma invasion of the brain. Penar P L; Khoshyomn S; Bhushan A; Tritton T R. (Division of Neurosurgery, University of Vermont College of Medicine, Burlington, USA.) NEUROSURGERY, (1997 Jan) 40 (1) 141-51. Journal code: NZL; 7802914. ISSN: 0148-396X. Pub. country: United States. Language: English.

AB OBJECTIVE: Glioblastoma multiforme is a malignant primary brain tumor associated with short patient survival despite aggressive treatment, in part because of its propensity to aggressively infiltrate into brain tissue. Glioblastoma multiforme is also unique because it is the only nonepithelial human tumor for which excessive activation of **epidermal growth factor receptor** (**EGFR**) has been consistently linked to tumor growth and patient survival, and **EGFR** activation promotes glioblastoma multiforme infiltration in vitro. METHODS: Cocultures of human glioblastoma spheroids (derived from three separate patients) and fetal rat brain aggregates were examined for infiltration using confocal microscopy, in the presence of 0 to 100 mumol/L genistein, a tyrosine kinase (TK) inhibitor, and 3 mumol/L tyrphostin A25, a specific **EGFR**-TK inhibitor. RESULTS: Infiltration (not attachment) was completely inhibited by genistein at 10 mumol/L, the IC20 for monolayer growth inhibition in two cell lines. Tyrphostin A25 at 3 mumol/L (the IC20 for monolayers) reduced invasion in a third cell line from 38.8 +/- 6.1% invasion-hour per hour (n = 5) to 2.9 +/- 1.2% invasion-hour per hour (n = 6) (P = 0.0002, two-tailed t test, 93% inhibition), and from 0.54 +/- 0.065% per hour (slope) to 0.028 +/- 0.018% per hour (P = 0.00001, 95% inhibition). Maximal percent invasion was reduced from 100 +/- 0 to 7.4 +/- 5.6% of the fetal rat brain aggregate. No change was detected in **EGFR**-associated **tyrosine phosphorylation** at those doses in monolayers by 32P immunolabeling, consistent with the known effects of low concentrations of TK inhibitors. An increase in expression of wild-type and truncated **EGFR** was demonstrated by Western blotting. Invasion was equally well inhibited by a monoclonal **antibody** to the high-affinity ligand binding domain of **EGFR** and not by **antibody** to an inactive domain. CONCLUSION: Our observations support the role of **EGFR** activation as a determinant by which

glioblastoma invades normal brain tissue, and we show that invasion can be effectively inhibited at much lower concentrations of TK inhibitors than are necessary for growth suppression.

L10 ANSWER 11 OF 13 MEDLINE DUPLICATE 8
96313262 Document Number: 96313262. PubMed ID: 8710366. Intracellular expression of a single-chain **antibody** directed to the **EGFR** leads to growth inhibition of tumor cells. Jannot C B; Beerli R R; Mason S; Gullick W J; Hynes N E. (Friedrich Miescher Institute, Basel, Switzerland.) ONCOGENE, (1996 Jul 18) 13 (2) 275-82. Journal code: ONC; 8711562. ISSN: 0950-9232. Pub. country: ENGLAND: United Kingdom. Language: English.

AB A gene encoding a single-chain **antibody** (scFv) which specifically binds the **epidermal growth factor receptor** (**EGFR**) has been constructed from hybridoma cells producing the R1 monoclonal **antibody**. The gene, designated scFv-R1R, was introduced into **EGFR** transformed NIH3T3 cells via retroviral infection. scFv-R1R was directed to the lumen of the endoplasmic reticulum (ER) where it bound the extracellular domain of the receptor inhibiting its appearance on the plasma membrane. In these cells, EGF induced **tyrosine phosphorylation** of the **EGFR** and several substrates was greatly reduced. Furthermore, intracellular retention of **EGFR** caused a partial inhibition in the transformed growth of the cells. Intracellular expression of receptor tyrosine kinase directed scFvs is a novel approach for affecting tumor cell growth. We have recently shown that scFv-5R directed to ErbB2, another member of the ErbB family, **blocks** the anchorage independent growth of ErbB2 transformed cells. In order to examine the effects of scFv-R1R and scFv-5R on the long-term growth of tumor cells overexpressing either **EGFR** or ErbB2, retroviruses encoding the two scFvs were used to infect various human tumor cell lines. Intracellular expression of the scFvs resulted in a marked inhibition of stable colony formation in some of the cell lines. In general, inhibition was observed when overexpressed receptor was targeted. However, in some cases expression of both scFvs was incompatible with long term cell growth suggesting that heterodimers of ErbB2 and **EGFR** are essential for the growth of some human tumor cell lines.

L10 ANSWER 12 OF 13 EMBASE COPYRIGHT 2001 ELSEVIER SCI. B.V.DUPLICATE 9
95283882 EMBASE Document No.: 1995283882. **Antibody**-induced inhibition of growth of **EGFR** overexpressing tumours occurs in the absence of receptor down-regulation. Modjtahedi H.; Dean C.. Institute of Cancer Research, Section of Immunology,Sutton, Surrey SM2 5NG, United Kingdom. International Journal of Oncology 7/4 (783-788) 1995. ISSN: 1019-6439. CODEN: IJONES. Pub. Country: Greece. Language: English. Summary Language: English.

AB Using two **antibodies** which bind to distinct epitopes on the extracellular domain of the EGF receptor (**EGFR**) we have developed a novel method for monitoring **EGFR** expression and the behaviour of monoclonal **antibody** (mAb) bound to the receptor. We have used this method to investigate the fate of the rat mAb ICR80 following binding to the EGF receptor on tumour cells. **Antibody** ICR80, which was raised against the external domain of the EGF receptor on a human brain tumour (A172) cell line and was employed in this study, has the following properties. It (a) **blocks** the binding of EGF, TGF.alpha. and HB-EGF to the **EGFR**, (b) prevents the EGF, TGF.alpha. and HB-EGF induced **tyrosine phosphorylation** of the **EGFR**, and (c) inhibits the growth in vitro of the head and neck tumour (HN5) cell line overexpressing the EGF receptor. Our

results presented herein also show that EGF receptor blockade by **antibody** ICR80 is not accompanied by detectable loss of **antibody** from the cell surface or downregulation of the receptor. On the basis of these results we conclude that the long-lasting blockade of the EGF receptor on tumour cells by **antibody** may be an important factor in preventing the binding of growth factors which are essential for their continued proliferation.

L10 ANSWER 13 OF 13 MEDLINE DUPLICATE 10
93179513 Document Number: 93179513. PubMed ID: 8382707. Anti-**epidermal growth factor receptor** monoclonal **antibodies** affecting signal transduction. Reins H A; Steinhilber G; Freiberg B; Anderer F A. (Friedrich-Miescher-Laboratorium der Max-Planck-Gesellschaft, Tuebingen, Federal Republic of Germany.) JOURNAL OF CELLULAR BIOCHEMISTRY, (1993 Feb) 51 (2) 236-48. Journal code: HNF; 8205768. ISSN: 0730-2312. Pub. country: United States. Language: English.

AB Monoclonal **antibodies** prepared against tyrosine phosphorylated **epidermal growth factor receptor** (**EGFR**) were tested for their effects on transmembrane signal transduction in A431 tumor cells. Monoclonal **antibodies** (mab) defined by SDS-sensitive epitopes, i.e., epitopes with conformational specificity, were most effective. Mab 5-125 reacting with a site of the extracellular **EGFR** domain blocked EGF-binding and cell proliferation in vitro, as well as tumor growth in vivo. However, this mab appeared not to be internalized upon binding to **EGFR** and did not trigger **EGFR** autophosphorylation. In contrast, mab 5-D43, also defined by an SDS-sensitive epitope and reacting with an extracellular **EGFR** site, did not **block** EGF binding but was readily internalized after binding to **EGFR** of untreated A431 cells. This mab induced **EGFR tyrosine phosphorylation** in cell lysates and tyrosine-specific autophosphorylation of insolubilized **EGFR** immune complexes. Cell growth in vitro was greatly stimulated in the presence of mab 5-D43. Since interaction of mab 5-D43 with **EGFR** induced most EGF-specific functions, although it did not bind to the EGF-specific site of **EGFR**, we have to assume that binding of mab 5-D43 to **EGFR** induced a conformational shift that activated the cytoplasmic **EGFR** kinase site. On the other hand, activation and/or accessibility of the **EGFR** kinase site could be blocked by mab 1-594, which is defined by an SDS-insensitive protein epitope of the cytoplasmic **EGFR** domain. Blocking of the **EGFR** kinase site by mab 1-594 also abolished EGF-induced **tyrosine phosphorylation** of endogenous cellular substrates with molecular masses of 145, 97, 85, 37, and 32 kDa, as well as of exogenous substrates such as GAT copolymer.

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(FILE 'HOME' ENTERED AT 08:36:19 ON 05 DEC 2001)

FILE 'MEDLINE, EMBASE, BIOSIS, SCISEARCH, CAPLUS' ENTERED AT 08:36:42 ON 05 DEC 2001

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L1      2245934 S ANTIBOD?
L2         7773 S L1 AND EPIDERMAL GROWTH FACTOR RECEPTOR
L3         2816 S L2 AND EGFR
L4            0 S L3 AND INHIBITS TYROSINE PHOSPHORYLATION
L5          217 S L3 AND TYROSINE PHOSPHORYLATION
L6           46 S L5 AND BLOCK
L7            5 S L6 AND INTERNALIZED
L8            0 S L7 AND DEGRADATION
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L9 1 DUP REMOVE L7 (4 DUPLICATES REMOVED)
L10 13 DUP REMOVE L6 (33 DUPLICATES REMOVED)
L11 0 S L10 AND 63 KD

=> s l3 and VEGF production

L12 5 L3 AND VEGF PRODUCTION

=> dup remove l12

PROCESSING COMPLETED FOR L12
L13 1 DUP REMOVE L12 (4 DUPLICATES REMOVED)

=> d l13 cbib abs

L13 ANSWER 1 OF 1 MEDLINE DUPLICATE 1
93184390 Document Number: 93184390. PubMed ID: 7680247. Epidermal growth factor stimulates vascular endothelial growth factor production by human malignant glioma cells: a model of glioblastoma multiforme pathophysiology. Goldman C K; Kim J; Wong W L; King V; Brock T; Gillespie G Y. (Brain Tumor Research Laboratories, Division of Neurosurgery, University of Alabama, Birmingham 35294-0006.) MOLECULAR BIOLOGY OF THE CELL, (1993 Jan) 4 (1) 121-33. Journal code: BAU; 9201390. ISSN: 1059-1524. Pub. country: United States. Language: English.

AB Hypervascularity, focal necrosis, persistent cerebral edema, and rapid cellular proliferation are key histopathologic features of glioblastoma multiforme (GBM), the most common and malignant of human brain tumors. By immunoperoxidase and immunofluorescence, we definitively have demonstrated the presence of vascular endothelial growth factor (VEGF) and **epidermal growth factor receptor** (**EGFr**) in five out of five human glioma cell lines (U-251MG, U-105MG, D-65MG, D-54MG, and CH-235MG) and in eight human GBM tumor surgical specimens. In vitro experiments with glioma cell lines revealed a consistent and reliable relation between **EGFr** activation and **VEGF production**; namely, EGF (1-20 ng/ml) stimulation of glioma cells resulted in a 25-125% increase in secretion of bioactive VEGF. Conditioned media (CM) prepared from EGF-stimulated glioma cell lines produced significant increases in cytosolic free intracellular concentrations of Ca2+ ([Ca2+]i) in human umbilical vein endothelial cells (HUVECs). Neither EGF alone or CM from glioma cultures prepared in the absence of EGF induced [Ca2+]i increases in HUVECs. Preincubation of glioma CM with A4.6.1, a monoclonal **antibody** to VEGF, completely abolished VEGF-mediated [Ca2+]i transients in HUVECs. Likewise, induction by glioma-derived CM of von Willebrand factor release from HUVECs was completely blocked by A4.6.1 pretreatment. These observations provide a key link in understanding the basic cellular pathophysiology of GBM tumor angiogenesis, increased vascular permeability, and cellular proliferation. Specifically, EGF activation of **EGFr** expressed on glioma cells leads to enhanced secretion of VEGF by glioma cells. VEGF released by glioma cells in situ most likely accounts for pathognomonic histopathologic and clinical features of GBM tumors in patients, including striking tumor angiogenesis, increased cerebral edema and hypercoagulability manifesting as focal tumor necrosis, deep vein thrombosis, or pulmonary embolism.

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FILE 'MEDLINE, EMBASE, BIOSIS, SCISEARCH, CAPLUS' ENTERED AT 08:36:42 ON 05 DEC 2001

L1 2245934 S ANTIBOD?
L2 7773 S L1 AND EPIDERMAL GROWTH FACTOR RECEPTOR
L3 2816 S L2 AND EGFR
L4 0 S L3 AND INHIBITS TYROSINE PHOSPHORYLATION
L5 217 S L3 AND TYROSINE PHOSPHORYLATION
L6 46 S L5 AND BLOCK
L7 5 S L6 AND INTERNALIZED
L8 0 S L7 AND DEGRADATION
L9 1 DUP REMOVE L7 (4 DUPLICATES REMOVED)
L10 13 DUP REMOVE L6 (33 DUPLICATES REMOVED)
L11 0 S L10 AND 63 KD
L12 5 S L3 AND VEGF PRODUCTION
L13 1 DUP REMOVE L12 (4 DUPLICATES REMOVED)

=> dup remove l3

PROCESSING IS APPROXIMATELY 61% COMPLETE FOR L3
PROCESSING COMPLETED FOR L3
L14 1055 DUP REMOVE L3 (1761 DUPLICATES REMOVED)

=> s l14 and threonine phosphorylation

L15 0 L14 AND THREONINE PHOSPHORYLATION

=> s l14 and threonine

L16 11 L14 AND THREONINE

=> dup remove l16

PROCESSING COMPLETED FOR L16
L17 11 DUP REMOVE L16 (0 DUPLICATES REMOVED)

=> d l17 1-11 cbib abs

L17 ANSWER 1 OF 11 MEDLINE
2000404731 Document Number: 20357377. PubMed ID: 10801894. Peroxynitrite targets the **epidermal growth factor receptor**, Raf-1, and MEK independently to activate MAPK. Zhang P; Wang Y Z; Kagan E; Bonner J C. (Laboratory of Pulmonary Pathobiology, NIEHS, National Institutes of Health, Research Triangle Park, North Carolina 27709, USA.) JOURNAL OF BIOLOGICAL CHEMISTRY, (2000 Jul 21) 275 (29) 22479-86. Journal code: HIV; 2985121R. ISSN: 0021-9258. Pub. country: United States. Language: English.
AB Activation of ERK-1 and -2 by H(2)O(2) in a variety of cell types requires **epidermal growth factor receptor** (**EGFR**) phosphorylation. In this study, we investigated the activation of ERK by ONOO(-) in cultured rat lung myofibroblasts. Western blot analysis using anti-phospho-ERK **antibodies** along with an ERK kinase assay using the phosphorylated heat- and acid-stable protein (PHAS-1) substrate demonstrated that ERK activation peaked within 15 min after ONOO(-) treatment and was maximally activated with 100 micrometer ONOO(-). Activation of ERK by ONOO(-) and H(2)O(2) was blocked by the antioxidant N-acetyl-l-cysteine. Catalase blocked ERK activation by H(2)O(2), but not by ONOO(-), demonstrating that the effect of ONOO(-) was not due to the generation of H(2)O(2). Both H(2)O(2) and ONOO(-) induced

phosphorylation of **EGFR** in Western blot experiments using an anti-phospho-**EGFR antibody**. However, the **EGFR** tyrosine kinase inhibitor AG1478 abolished ERK activation by H(2)O(2), but not by ONOO(-). Both H(2)O(2) and ONOO(-) activated Raf-1. However, the Raf inhibitor forskolin blocked ERK activation by H(2)O(2), but not by ONOO(-). The MEK inhibitor PD98059 inhibited ERK activation by both H(2)O(2) and ONOO(-). Moreover, ONOO(-) or H(2)O(2) caused a cytotoxic response of myofibroblasts that was prevented by preincubation with PD98059. In a cell-free kinase assay, ONOO(-) (but not H(2)O(2)) induced autophosphorylation and nitration of a glutathione S-transferase-MEK-1 fusion protein. Collectively, these data indicate that ONOO(-) activates **EGFR** and Raf-1, but these signaling intermediates are not required for ONOO(-)-induced ERK activation. However, MEK-1 activation is required for ONOO(-)-induced ERK activation in myofibroblasts. In contrast, H(2)O(2)-induced ERK activation is dependent on **EGFR** activation, which then leads to downstream Raf-1 and MEK-1 activation.

L17 ANSWER 2 OF 11 MEDLINE

2001039855 Document Number: 20329466. PubMed ID: 10873065. Modulation of radiation response after **epidermal growth factor receptor** blockade in squamous cell carcinomas: inhibition of damage repair, cell cycle kinetics, and tumor angiogenesis. Huang S M; Harari P M. (Department of Human Oncology, University of Wisconsin School of Medicine and Comprehensive Cancer Center, Madison 53792-0600, USA.) CLINICAL CANCER RESEARCH, (2000 Jun) 6 (6) 2166-74. Journal code: C2H. ISSN: 1078-0432. Pub. country: United States. Language: English.

AB We have recently demonstrated that molecular blockade of the **epidermal growth factor receptor** with the anti-**epidermal growth factor receptor** (**EGFR**) monoclonal **antibody** C225 enhances the in vitro radiosensitivity of human squamous cell carcinomas (SCCs) derived from the head and neck. In the present study, we further investigated the capacity of C225 to modulate the in vitro and in vivo radiation response of human SCC tumor cells and xenografts, and we examined several potential mechanisms that may contribute to the enhanced radiation response induced by C225. Tumor xenograft studies demonstrated complete regression of both newly established (20 mm3) and well-established (100 mm3) SCC tumors over a 55-100 day follow-up period in athymic mice treated with the combination of C225 (i.p. injection) and radiation. Cell cycle analysis via flow cytometry confirmed that combined treatment with C225 and radiation induced an accumulation of cells in the more radiosensitive cell cycle phases (G1, G2-M) with concurrent reduction in the proportion of cells in the more radioresistant S phase. Results from sublethal damage repair and potentially lethal damage repair analyses in cultured SCC cells demonstrated a strong inhibitory effect of C225 on postradiation damage repair. Further, exposure of SCC cells to C225 induced a redistribution of DNA-dependent protein kinase from the nucleus to the cytosol, suggesting one potential mechanism whereby C225 may influence the cellular response to radiation. Immunohistochemical analysis of SCC tumor xenografts after systemic administration of C225 demonstrated inhibition of the in vivo expression of tumor angiogenesis markers, including vascular endothelial growth factor and Factor VIII. Taken together, the collective data suggest that the profound in vivo antitumor activity identified in the xenograft setting when C225 is combined with radiation derives from more than simply the antiproliferative and cell cycle effects of **EGFR** system inhibition. In addition to antiproliferative growth inhibition, **EGFR** blockade with C225

appears to influence the capacity of human SCCs to effect DNA repair after exposure to radiation, and to express classic markers of tumor angiogenesis.

L17 ANSWER 3 OF 11 MEDLINE

1999232131 Document Number: 99232131. PubMed ID: 10216485. Investigation of the Mek-MAP kinase-Rsk pathway in human breast cancer. Salh B; Marotta A; Matthewson C; Ahluwalia M; Flint J; Owen D; Pelech S. (Department of Medicine, University of British Columbia, Vancouver, Canada.) ANTICANCER RESEARCH, (1999 Jan-Feb) 19 (1B) 731-40. Journal code: 59L; 8102988. ISSN: 0250-7005. Pub. country: Greece. Language: English.

AB BACKGROUND: Mitogenic signaling through the principal growth factor receptor tyrosine kinase (RTK) pathway, i.e. RTK-->Ras-->Raf-->Mek-->MAPK has been implicated in the pathogenesis of human cancer. However, biochemical characterization of this has not been adequately assessed in human cancers. MATERIALS AND METHODS: Using extracts from 23 human breast cancers and control tissue from the same resected specimens, the protein levels, phosphotransferase activities and subcellular locations of the mitogen-activated protein (MAP) kinase isoforms p42 Erk2 and p44 Erk1 were examined, together with their phosphotransferase activities towards myelin basic protein (MBP) and a peptide substrate patterned after the Thr-669 site in the **epidermal growth factor receptor** (**EGFR** T669) that is phosphorylated by MAP kinase. RESULTS: Overexpression of both Erk2 and Erk1 isoforms was evident using specific **antibodies**. A universal activation of MBP and **EGFR** T669 peptide phosphotransferase activities was also found (up to 3-fold). MonoQ fractionation resolved the bulk of the **EGFR** T669 peptide phosphorylation from elution of the MAP kinase protein. Erk1 and Erk2 activities determined by specific immunoprecipitation were increased by up to only 2.5-fold in only 50% of tumors overall. Immunohistochemical studies, using a monoclonal **antibody** specific for Erk2 demonstrated that the cellular distribution of this MAP kinase was similar in both control and tumor tissues, and Erk2 was largely confined to normal and malignant acini, whilst the intensity of staining was actually reduced in the tumor tissue. Mek1 and especially Mek2 protein expression, as well as MAP kinase kinase activity as determined by phosphorylation of kinase-inactive Erk [GST-K71A] were increased in cancer samples. CONCLUSIONS: a) This confirms that MAP kinase activity is increased in human breast cancer. However, the frequency and magnitude of this change is dependent upon the chosen methodology (i.e. crude lysate assays versus specific immunoprecipitation). b) A MAP-kinase-independent source of increased **EGFR** T669 phosphotransferase activity in tumor extracts has been demonstrated for the first time in human breast cancer. c) By immunohistochemistry, Erk2 protein was actually found to exhibit lower intensity in tumor samples; the increased expression was most likely due to its increased distribution. d) Increased Mek protein expression and activation have been demonstrated for the first time in human breast tumors.

L17 ANSWER 4 OF 11 MEDLINE

1998104141 Document Number: 98104141. PubMed ID: 9430697. Physical interaction between **epidermal growth factor receptor** and DNA-dependent protein kinase in mammalian cells. Bandyopadhyay D; Mandal M; Adam L; Mendelsohn J; Kumar R. (Department of Clinical Investigation, University of Texas M. D. Anderson Cancer Center, Houston 77030, USA.) JOURNAL OF BIOLOGICAL CHEMISTRY, (1998 Jan 16) 273 (3) 1568-73. Journal code: HIV; 2985121R. ISSN: 0021-9258. Pub. country:

United States. Language: English.
AB Binding of extracellular ligands to **epidermal growth factor receptors** (**EGFR**) activate signal transduction pathways associated with cell proliferation, and these events are inhibited by monoclonal **antibodies** against **EGFR**. Since efficient DNA repair in actively growing cells may require growth factor signaling, it was of interest to explore any linkage between **EGFR**-mediated signaling and DNA-dependent protein kinase (DNA-PK), an enzyme believed to be involved in repairing double strand breaks and V(D)J recombination. We report that anti-**EGFR** monoclonal **antibodies** (mAbs), and not **EGFR** ligands, trigger a specific early physical interaction between **EGFR** and a 350-kDa catalytic subunit of DNA or its regulatory heterodimeric complex Ku70/80, in a variety of cell types, both in vivo and in vitro. Inhibition of **EGFR** signaling by anti-**EGFR** mAb was accompanied by a reduction in the levels of the DNA-PK and its activity in the nuclear fraction. Confocal imaging revealed that a substantial amount of DNA-PK was co-localized with **EGFR** in anti-**EGFR** mAb-treated cells. Anti-**EGFR** mAb-induced physical interaction between **EGFR** and DNA-PK or Ku70/80 was dependent on the presence of **EGFR**, but not on the levels of **EGFR**. The **EGFR** associated with DNA-PK or Ku70/80 retains its intrinsic kinase activity. Our findings demonstrate the existence of a novel cellular pathway in mammalian cells that involves physical interactions between **EGFR** and DNA-PK or Ku70/80 in response to inhibition of **EGFR** signaling. Our present observations suggest a possible role of **EGFR** signaling in maintenance of the nuclear levels of DNA-PK, and interference in **EGFR** signaling may possibly result in the impairment of DNA repair activity in the nuclei in anti-**EGFR** mAb-treated cells.

L17 ANSWER 5 OF 11 MEDLINE
1998223363 Document Number: 98223363. PubMed ID: 9563874. Interactions between the **epidermal growth factor receptor** and type I protein kinase A: biological significance and therapeutic implications. Ciardiello F; Tortora G. (Dipartimento di Endocrinologia e Oncologia Molecolare e Clinica, Facolta di Medicina e Chirurgia, Universita di Napoli Federico II, Italy.) CLINICAL CANCER RESEARCH, (1998 Apr) 4 (4) 821-8. Ref: 78. Journal code: C2H; 9502500. ISSN: 1078-0432. Pub. country: United States. Language: English.
AB Peptide growth factors regulate normal cellular proliferation and differentiation through autocrine and paracrine pathways and are involved in cancer development and progression. Among the endogenous growth factors, the epidermal growth factor (EGF)-related proteins play an important role in the pathogenesis of human cancer. In fact, overexpression of EGF-related growth factors such as transforming growth factor alpha and amphiregulin and/or their specific receptor, the EGF receptor (**EGFR**), has been detected in several types of human cancers, including breast, lung, and colorectal cancers. Therefore, the blockade of **EGFR** activation by using anti-**EGFR** monoclonal **antibodies** (MAbs) has been proposed as a potential anticancer therapy. The cAMP-dependent protein kinase (PKA) is an intracellular enzyme with serine-**threonine** kinase activity that plays a key role in cell growth and differentiation. Two PKA isoforms with identical catalytic (C) subunits but different cAMP-binding regulatory (R) subunits (defined as RI in PKAI and RII in PKAII) have been identified. Predominant expression of PKAII is found in normal nonproliferating tissues and in growth-arrested cells, whereas enhanced levels of PKAI are detected steadily in tumor cells and transiently in normal cells exposed to mitogenic stimuli. Overexpression of PKAI has been correlated recently with poor prognosis in breast cancer patients. Inhibition of PKAI

expression and function by specific pharmacological agents such as the selective cAMP analogue 8-chloro-cAMP (8-Cl-cAMP) induces growth inhibition in various human cancer cell lines in vitro and in vivo. We have provided experimental evidence of a functional cross-talk between ligand-induced **EGFR** activation and PKAI expression and function. In fact, PKAI is overexpressed and activated following transforming growth factor alpha-induced transformation in several rodent and human cell line models. Furthermore, PKAI is involved in the intracellular mitogenic signaling following ligand-induced **EGFR** activation. We have shown that an interaction between **EGFR** and PKAI occurs through direct binding of the RI subunit to the Grb2 adaptor protein. In this respect, PKAI seems to function downstream of the **EGFR**, and experimental evidence suggests that PKAI is acting upstream of the mitogen-activated protein kinase pathway. We have also demonstrated that the functional interaction between the **EGFR** and the PKAI pathways could have potential therapeutic implications. In fact, the combined interference with both **EGFR** and PKAI with specific pharmacological agents, such as anti-**EGFR** blocking MAbs and cAMP analogues, has a cooperative antiproliferative effect on human cancer cell lines in vitro and in vivo. The antitumor activity of this combination could be explored in a clinical setting because both the 8-Cl-cAMP analogue and the anti-**EGFR** blocking MAb C225 have entered human clinical trial evaluation. Finally, both MAb C225 and 8-Cl-cAMP are specific inhibitors of intracellular mitogenic signaling that have different mechanisms of action compared with conventional cytotoxic drugs. In this respect, a cooperative growth-inhibitory effect in combination with several chemotherapeutic agents in a large series of human cancer cell lines in vitro and in vivo has been demonstrated for anti-**EGFR** blocking MAbs or for 8-Cl-cAMP. Therefore, the combination of MAb C225 and 8-Cl-cAMP following chemotherapy could be investigated in cancer patients.

L17 ANSWER 6 OF 11 SCISEARCH COPYRIGHT 2001 ISI (R)
96:673872 The Genuine Article (R) Number: VG672. MUTATIONAL ANALYSIS OF THE NUCLEOTIDE-BINDING SITE OF THE **EPIDERMAL GROWTH-FACTOR RECEPTOR** AND V-SRC PROTEIN-TYROSINE KINASES.
CHAN C L; GILL G N (Reprint). UNIV CALIF SAN DIEGO, DEPT MED, 9500 GILMAN DR, LA JOLLA, CA, 92093 (Reprint); UNIV CALIF SAN DIEGO, DEPT MED, LA JOLLA, CA, 92093. JOURNAL OF BIOLOGICAL CHEMISTRY (13 SEP 1996) Vol. 271, No. 37, pp. 22619-22623. ISSN: 0021-9258. Pub. country: USA. Language: ENGLISH.
ABSTRACT IS AVAILABLE IN THE ALL AND IALL FORMATS
AB Tyrosine kinases differ from serine/**threonine** kinases in sequences located at the active site where ATP and substrate bind. In the structure of cyclic AMP-dependent protein kinase, the catalytic loop contains the sequence Lys-Pro-Glu where the Lys residue contacts the gamma-phosphate of ATP and the Glu residue contacts a basic residue located in the peptide substrate. In tyrosine kinases, the analogous sequence is Ala-Ala-Arg in the receptor tyrosine kinase subfamily and Arg-Ala-Ala in the Src tyrosine kinase subfamily. To deduce the role of these residues in tyrosine kinase function, site-directed mutations were prepared in the **epidermal growth factor receptor** (**EGFR**) and in v-Src and effects on ATP binding and kinase activity were determined. Changing Arg to either Lys or Ala dramatically reduced activity of both tyrosine kinases and this correlated with loss of ATP binding. Changing the orientation of this sequence impaired activity of **EGFR** to a greater extent than that of v-Src but did not change substrate specificity of the two enzymes. These results support the hypothesis that Arg functions to coordinate the

gamma-phosphate of ATP. Analysis of sequence inversions in the catalytic loop indicate that the active site of v-Src exhibits greater flexibility than that of **EGFR**.

L17 ANSWER 7 OF 11 MEDLINE
96328084 Document Number: 96328084. PubMed ID: 8706005. Anti-**epidermal growth factor receptor** monoclonal **antibody** 225 up-regulates p27KIP1 and induces G1 arrest in prostatic cancer cell line DU145. Peng D; Fan Z; Lu Y; DeBlasio T; Scher H; Mendelsohn J. (Laboratory of Receptor Biology, Memorial Sloan-Kettering Cancer Center, New York, New York 10021, USA.) CANCER RESEARCH, (1996 Aug 15) 56 (16) 3666-9. Journal code: CNF; 2984705R. ISSN: 0008-5472. Pub. country: United States. Language: English.

AB Autocrine production of transforming growth factor alpha and overexpression of the **epidermal growth factor receptor** (**EGFR**) may contribute to androgen-independent prostatic cancer growth at both primary and metastatic sites. Previously, we showed that human **EGFR**-blocking monoclonal **antibody** mAb225 inhibited the growth of DU145 human prostatic cancer cells. Here we explore the hypothesis that mAb225 may act by interfering with cell cycle traversal in these cells. Treatment with mAb225 induced G1 arrest, which was accompanied by a marked decrease in CDK2-, cyclin A-, and cyclin E-associated histone H1 kinase activities, and a sustained increase in cell cycle inhibitor p27KIP1. The increased p27KIP1 levels were attributable to elevation of both transcription and translation. CDK2 associated with p27KIP1 was increased in mAb225-treated DU145 cells. The retinoblastoma-related protein p130 remained hypophosphorylated in these retinoblastoma-negative cells. These studies demonstrate that the antiproliferative effect of **EGFR** blockade in DU145 cells may be mediated by up-regulation of p27KIP1 at both the mRNA and protein levels.

L17 ANSWER 8 OF 11 MEDLINE
97099431 Document Number: 97099431. PubMed ID: 8944008. Antitumor activity of combined blockade of **epidermal growth factor receptor** and protein kinase A. Ciardiello F; Damiano V; Bianco R; Bianco C; Fontanini G; De Laurentiis M; De Placido S; Mendelsohn J; Bianco A R; Tortora G. (Dipartimento di Endocrinologia e Oncologia Molecolare e Clinica, Universita degli Studi di Napoli Federico II, Naples, Italy.) JOURNAL OF THE NATIONAL CANCER INSTITUTE, (1996 Dec 4) 88 (23) 1770-6. Journal code: J9J; 7503089. ISSN: 0027-8874. Pub. country: United States. Language: English.

AB BACKGROUND: Epidermal growth factor (EGF)-related proteins, such as transforming growth factor-alpha (TGF-alpha), control cancer cell growth through hormonal pathways (i.e., autocrine [hormone acts on cell that produces it] and paracrine [hormone acts on nearby cells] pathways). Overexpression of TGF-alpha and/or its receptor (**EGFR**) has been detected in human cancers. The blockade of **EGFR** activation by the use of anti-**EGFR** monoclonal **antibodies** (MAbs) has been proposed as a potential anticancer therapy. The type I cyclic adenosine monophosphate (cAMP)-dependent protein kinase (PKAI) is generally overexpressed in human cancer cells and is involved in neoplastic transformation. Inhibition of PKAI by selective cAMP analogues, such as 8-chloro-cAMP (8-CI-cAMP), induces growth inhibition in various human cancer cell lines. PURPOSE: On the basis of our previous observations of a cooperative anti-proliferative effect of anti-**EGFR** Mab 528 and 8-Cl-cAMP in human cancer cell lines in vitro, we evaluated the anticancer activity in vivo of the combination of an anti-**EGFR** MAb (MAb C225) and 8-Cl-cAMP. METHODS: Athymic mice were injected subcutaneously with 10(7) human colon carcinoma GEO cells. After 7 days, when established tumor xenografts of 0.30-0.35 cm3 were detectable, 10-15 mice per group were treated intraperitoneally twice

weekly with different doses of 8-Cl-cAMP and/or MAb C225. Cancer cell expression of various growth factors was evaluated by immunohistochemical analysis in tumors obtained from control and treated mice. Data were evaluated for statistical significance using the Student's t test and the Mantel-Cox logrank test. All P values represent two-sided tests of statistical significance. RESULTS: A 5-week treatment with low doses of 8-Cl-cAMP (0.5 mg/dose) and MAb C225 (0.25 mg/dose) blocked GEO tumor growth (compared with that in control mice; P < .00001) and suppressed cancer cell production of autocrine growth factors, such as TGF-alpha, amphiregulin, and CRIPTO, and of angiogenic (promotes new blood vessel formation) factors, such as vascular endothelial growth factor and basic fibroblast growth factor, with no signs of toxicity. Control and 8-Cl-cAMP (0.5 mg/dose)-treated mice died within 9-10 weeks after tumor cell injection. In MAb C225 (0.25 mg/dose)-treated mice, GEO tumors resumed a growth rate comparable to that in control animals within 3 weeks following the end of treatment and the mice died between 11 and 20 weeks after tumor cell injection. GEO tumor growth was significantly delayed in the MAb C225 plus 8-Cl-cAMP treatment group (P < .00001) and was accompanied by a prolonged survival of mice (P < .00001) as compared with the control group. CONCLUSIONS: Long-term treatment with a combination of agents that selectively inhibit two intracellular signal-transduction enzymes, such as the PKAI serine-**threonine** kinase and the **EGFR** tyrosine kinase, has anticancer activity in vivo, reflected by suppression of tumor proliferation and angiogenesis, with no signs of toxicity. IMPLICATIONS: Since these inhibitors of intracellular mitogenic (growth-stimulating) signaling have a different mechanism(s) of action and do not antagonize the effects of cytotoxic therapy, a combination of anti-**EGFR** MAb C225 and 8-Cl-cAMP should be investigated as a nontoxic, long-term treatment for cancer patients following chemotherapy.

L17 ANSWER 9 OF 11 MEDLINE

96183364 Document Number: 96183364. PubMed ID: 8610433. The protein kinase activity of the large subunit of herpes simplex virus type 2 ribonucleotide reductase (ICP10) fused to the extracellular domain of the **epidermal growth factor receptor** is ligand-inducible. Smith C C; Luo J H; Aurelian L. (Department of Pharmacology, University of Maryland School of Medicine, Baltimore 21201, USA.) VIROLOGY, (1996 Mar 15) 217 (2) 425-34. Journal code: XEA; 0110674. ISSN: 0042-6822. Pub. country: United States. Language: English.

AB The gene coding for the large subunit of herpes simplex virus type 2 ribonucleotide reductase (RR) (ICP10) has a unique 5' terminal domain the product of which has a serine/**threonine** (Ser/Thr) protein kinase (PK) catalytic domain preceded by a transmembrane (TM) segment. Because ICP10 localizes on the cell surface and is internalized by the endocytic pathway like an activated growth factor receptor (Hunter et al., 1995, Virology 210, 345-360), we asked whether it is ligand-inducible in order to examine whether it has intrinsic transphosphorylating activity. We constructed a chimeric expression vector that contains the extracellular and TM domains of the **epidermal growth factor receptor** (**EGFR**) joined to the intracellular PK and RR domains of ICP10 (pCH5) and established constitutively expressing cell lines in NIH3T3 2.2 cells that do not express **EGFR**. The chimeric protein, designated p210 CH5, localized to the surface of these cells as determined by immunofluorescent staining with MAb **EGFR**, and it bound 125I-EGF.p210 CH5 coprecipitated with protein species p170, p120, p88, p60, p44, p34, and p25. EGF treatment activated the PK activity of p210 CH5, resulting in its autophosphorylation and the phosphorylation of the p120, p88, and p34 species. Immunoprecipitation/immunoblotting with

anti-ras-GAP **antibody** and phosphoamino acid analysis indicated that p120 is ras-GAP and it is phosphorylated on Ser/Thr residues. The identities of the phosphorylated p88 and p34 are still unknown. The data indicate that when fused to a ligand-regulated extracellular domain (**EGFR**), the ICP10 PK auto- and transphosphorylating activities are ligand-inducible. These findings support the interpretation that the ICP10 PK activity is intrinsic and indicate that ras-GAP is one of its phosphorylation substrates.

L17 ANSWER 10 OF 11 MEDLINE

1999034801 Document Number: 99034801. PubMed ID: 9815969. Cooperative antiproliferative effects of 8-chloro-cyclic AMP and 528 anti-**epidermal growth factor receptor** monoclonal **antibody** on human cancer cells. Ciardiello F; Damiano V; Bianco C; di Isernia G; Ruggiero A; Caraglia M; Tagliaferri P; Baselga J; Mendelsohn J; Bianco A R; +. (Cattedra di Oncologia Medica, Facolta di Medicina e Chirurgia, Universita degli Studi di Napoli Federico II, Via S. Pansini 5, 80131 Napoli, Italy.) CLINICAL CANCER RESEARCH, (1995 Feb) 1 (2) 161-7. Journal code: C2H; 9502500. ISSN: 1078-0432. Pub. country: United States. Language: English.

AB 8-Chloro-cyclic AMP (8-Cl-cAMP), a site-selective cAMP analogue, is a specific inhibitor of type I cAMP-dependent protein kinase (PKAI) and induces growth inhibition in several human and rodent tumor cell lines. The anti-**epidermal growth factor receptor** (**EGFR**) mAb 528 is a blocking **antibody** able to inhibit the in vitro and in vivo growth of several human cancer cell lines that express functional **EGFRs**. Since enhanced levels of PKAI are generally found in tumor cells and an increase in PKAI expression is induced by transformation through a transforming growth factor alpha/**EGFR** autocrine pathway, we have evaluated whether treatment with mAb 528 in combination with 8-Cl-cAMP may have an additive or synergistic growth inhibitory effect on human cancer cells. A dose-dependent inhibition of monolayer cell growth was observed in two human colon cancer cell lines (GEO and CBS) and in a human breast cancer cell line (MDA-468) by treatment with either mAb 528 or 8-Cl-cAMP with 50% inhibitory concentration of 2-10 microgram/ml or 20-25 micrometer, respectively. The combined treatment with low noninhibitory doses of mAb 528 (0.25 microgram/ml) and with 8-Cl-cAMP had a more than additive growth inhibitory effect with a 3- to 5-fold reduction in the 8-Cl-cAMP 50% inhibitory concentration in all cell lines tested. This combined treatment was similarly effective in inhibiting the soft agar cloning efficiency of GEO cells. 8-Cl-cAMP treatment of GEO cells induced a dose-dependent increase in cell membrane-associated **EGFRs** with a maximum 3- to 4-fold increase within 48-72 h of treatment. These results suggest that a double blockade of the PKAI serine-**threonine** kinase-dependent and of the **EGFR** tyrosine kinase-dependent pathways is potentially useful in cancer therapy.

L17 ANSWER 11 OF 11 MEDLINE

90037242 Document Number: 90037242. PubMed ID: 2553748. Signal transduction by epidermal growth factor occurs through the subclass of high affinity receptors. Defize L H; Boonstra J; Meisenhelder J; Kruijer W; Tertoolen L G; Tilly B C; Hunter T; van Bergen en Henegouwen P M; Moolenaar W H; de Laat S W. (Hubrecht Laboratory, Netherlands Institute for Developmental Biology, Utrecht.) JOURNAL OF CELL BIOLOGY, (1989 Nov) 109 (5) 2495-507. Journal code: HMV; 0375356. ISSN: 0021-9525. Pub. country: United States. Language: English.

AB Many cell types display two classes of **epidermal growth factor receptor** (**EGFR**) as judged from EGF

binding studies; i.e., a major class of low affinity **EGFR** and a minor class of high affinity **EGFR**. We have studied their respective contribution to the cascade of events elicited by EGF in human A431 carcinoma cells, using anti-**EGFR** mAb 2E9. This **antibody** specifically blocks EGF binding to low affinity **EGFR**, without activating receptors in intact cells, and thus enables us to study the effects of exclusive EGF binding to high affinity **EGFR**. We show that blocking of low affinity **EGFR** by mAb 2E9 has almost no effect on the activation of the receptor protein-tyrosine kinase by EGF, suggesting that **EGFR** kinase activation occurs exclusively through the subclass of high affinity **EGFR** (5-10%). In addition, we provide evidence that high affinity **EGFR** exists both in monomeric and dimeric forms, and that cross-phosphorylation of low affinity **EGFR** by high affinity **EGFR** may take place in dimers of both receptor types. We demonstrate that the following early cellular response to EGF are also unimpaired in the presence of mAb 2E9: (a) inositol phosphate production, (b) release of Ca2+ from intracellular stores, (c) rise in intracellular pH, (d) phosphorylation of EGF on **threonine** residue 654, (e) induction of c-fos gene expression, and (f) alteration in cell morphology.

As possible nonspecific side effects, we observed that the EGF induced Ca2+ influx and fluid-phase pinocytosis were inhibited in A431 cells in the presence of mAb 2E9. We conclude, therefore, that the activation of the **EGFR** signal transduction cascade can occur completely through exclusive binding of EGF to the subclass of high affinity **EGFR**.

=> s A431 cells

L18 5638 A431 CELLS

=> s l18 and EGFR antibody

L19 39 L18 AND EGFR ANTIBODY

=> dup remove l19

PROCESSING COMPLETED FOR L19
L20 10 DUP REMOVE L19 (29 DUPLICATES REMOVED)

=> d l20 1-10 cbib abs

L20 ANSWER 1 OF 10 MEDLINE DUPLICATE 1
2001462180 Document Number: 21397961. PubMed ID: 11507066.
Growth-inhibitory effect of a streptococcal antitumor glycoprotein on human epidermoid carcinoma **A431 cells**: involvement of dephosphorylation of epidermal growth factor receptor. Yoshida J; Ishibashi T; Nishio M. (Department of Pharmacology, Kanazawa Medical University, Ishikawa 920-0293, Japan.. yayuyo@kanazawa-med.ac.jp) . CANCER
RESEARCH, (2001 Aug 15) 61 (16) 6151-7. Journal code: CNF; 2984705R. ISSN: 0008-5472. Pub. country: United States. Language: English.
AB An antitumor glycoprotein [streptococcal acidic glycoprotein (SAGP)] purified from an extract of Streptococcus pyogenes inhibited the growth of human epidermoid carcinoma **A431 cells** overexpressing epidermal growth factor receptor (EGFR) in a time- and a concentration-dependent manner. The antiproliferative effect of SAGP was diminished by preincubating the cells with pertussis toxin and by coadministration of sodium orthovanadate, an inhibitor of protein tyrosine

phosphatases (PTPases). Western blot analysis showed that the immunoreactivity of a M(r) 170,000 band of cell lysate to antiphosphotyrosine antibody was reduced by SAGP, and the effect was abolished by sodium orthovanadate. The phosphotyrosine level of the precipitant with anti-**EGFR antibody** was reduced by SAGP, which was abolished by preincubation with pertussis toxin or by a coadministration with sodium orthovanadate. The PTPase activity transiently increased in the lysate of cells incubated with SAGP and was inhibitable by sodium orthovanadate. Additionally, preincubation of serum-starved **A431 cells** with SAGP decreased the epidermal growth factor-induced tyrosine phosphorylation of EGFR, and the effect of SAGP was sodium orthovanadate sensitive. These findings indicate that dephosphorylation of the M(r) 170,000 EGFR by activation of PTPase(s) may be responsible in part for the antiproliferative effect of SAGP on **A431 cells**.

L20 ANSWER 2 OF 10 MEDLINE DUPLICATE 2
2001374563 Document Number: 21324330. PubMed ID: 11431346. Acquired resistance to the antitumor effect of epidermal growth factor receptor-blocking antibodies in vivo: a role for altered tumor angiogenesis. Viloria-Petit A; Crombet T; Jothy S; Hicklin D; Bohlen P; Schlaeppi J M; Rak J; Kerbel R S. (Molecular and Cellular Biology Research, Sunnybrook and Women's College Health Sciences Centre, Toronto, Ontario M4N 3M5, Canada.) CANCER RESEARCH, (2001 Jul 1) 61 (13) 5090-101.
Journal code: CNF; 2984705R. ISSN: 0008-5472. Pub. country: United States.
Language: English.

AB Inhibitors of epidermal growth factor receptor (EGFR) signaling are among the novel drugs showing great promise for cancer treatment in the clinic. However, the possibility of acquired resistance to such drugs because of tumor cell genetic instabilities has not yet been explored. Here we report the experimental derivation and properties of such cell variants obtained from recurrent tumor xenografts of the human A431 squamous cell carcinoma, after two consecutive cycles of therapy with one of three different anti-EGFR monoclonal antibodies: mR3, hR3, or C225. Initial response to a 2-week period of treatment was generally total tumor regression and was not significantly different among the three antibody groups. However, tumors often reappeared at the site of inoculation, generally after prolonged latency periods, and most of the tumors became refractory to a second round of therapy. Cell lines established from such resistant tumors retained high EGFR expression, normal sensitivity to anti-**EGFR antibody** or ligand, and unaltered growth rate when compared with the parental line in vitro. In contrast, the A431 cell variants exhibited an accelerated growth rate and a significantly attenuated response to anti-**EGFR antibodies** in vivo relative to the parental line. Because of the reported suppressive effect of EGFR inhibitors on vascular endothelial growth factor (VEGF) expression, and the demonstrated role of VEGF in the angiogenesis and growth of A431 tumor xenografts, relative VEGF expression was examined. Five of six resistant variants expressed increased levels of VEGF, which paralleled an increase in both angiogenic potential in vitro and tumor angiogenesis in vivo. In addition, elevated expression of VEGF in variants of **A431 cells** obtained by gene transfection rendered the cells significantly resistant to anti-**EGFR antibodies** in vivo. Taken together, the results suggest that, at least in the A431 system, variants displaying acquired resistance to anti-**EGFR antibodies** can emerge

in vivo and can do so, at least in part, by mechanisms involving the selection of tumor cell subpopulations with increased angiogenic potential.

L20 ANSWER 3 OF 10 SCISEARCH COPYRIGHT 2001 ISI (R)
2001:13099 The Genuine Article (R) Number: 386QR. Attachment of A-431 cells on immobilized antibodies to the EGF receptor promotes cell spreading and reorganization of the microfilament system. Are A; Pinaev G; Burova E; Lindberg U (Reprint). Univ Stockholm, Wenner Gren Inst, Dept Cell Biol, S-10691 Stockholm, Sweden (Reprint); Russian Acad Sci, Inst Cytol, Dept Cell Culture, St Petersburg 194064, Russia; Russian Acad Sci, Inst Cytol, Dept Physiol Cell Cycle, St Petersburg 194064, Russia. CELL MOTILITY AND THE CYTOSKELETON (JAN 2001) Vol. 48, No. 1, pp. 24-36. Publisher: WILEY-LISS. DIV JOHN WILEY & SONS INC, 605 THIRD AVE, NEW YORK, NY 10158-0012 USA. ISSN: 0886-1544. Pub. country: Sweden; Russia. Language: English.
ABSTRACT IS AVAILABLE IN THE ALL AND IALL FORMATS
AB EGF-like sequences, inherent in a number of extracellular matrix proteins, participate in cell adhesion. It is possible that interactions of these sequences with EGF receptors (EGFR) affect actin filament organization. It was shown previously [Khrebtukova et al., 1991. Exp. Cell Res. 194:48-55] that antibodies specific to EGFR induce capping of these receptors and redistribution of cytoskeletal proteins in A-431 cells. Here we report that A-431 cells attach and spread on solid substrata coated with antibodies to EGFR, even in the absence of serum. Thus, EGFR can act as an adhesion protein and promote microfilament reorganization. Binding of the cells to the **EGFR-antibody** resulted in the formation of a unique cell shape characterized by numerous, actin-based filopodia radiating from the cell body, but without membrane ruffles. There was also a conspicuous circular belt of actin-containing fibers inside the cell margin, and many irregular actin aggregates in the perinuclear area. The morphologies and actin distributions in A-431 cells spread on fibronectin or laminin 2/4 were very different. On fibronectin, cells had polygonal shapes with numerous stress-fibers and thick actin-containing fibers along the cell edges. On laminin-covered substrata, the cells became fusiform and acquired broad leading lamellae with ruffles. In these cells, there were also a few bundles of filaments running the whole length of the cell body, and shorter bundles extending through the leading lamellae tot-yards the membrane ruffles in the cell edge. These effects and those seen with immobilized EGF suggest that different ligand/receptor complexes induce specific reorganizations of the microfilament system. Cell Motil. Cytoskeleton 48:24-36, 2001. (C) 2001 Wiley-Liss, Inc.

L20 ANSWER 4 OF 10 MEDLINE DUPLICATE 3
2000420980 Document Number: 20286525. PubMed ID: 10825287. Integrin alpha 2 beta 1-dependent EGF receptor activation at cell-cell contact sites. Yu X; Miyamoto S; Mekada E. (Institute of Life Science and Research Center for Innovative Cancer Therapy, Kurume University, Kurume, Fukuoka 839-0861, Japan.) JOURNAL OF CELL SCIENCE, (2000 Jun) 113 (Pt 12) 2139-47. Journal code: HNK; 0052457. ISSN: 0021-9533. Pub. country: ENGLAND: United Kingdom. Language: English.
AB Certain integrins including alpha 2 beta 1 and alpha 3 beta 1 localize to intercellular binding sites, and thus may participate in cell-cell interactions. We demonstrated here the physical and functional associations of integrin alpha 2 beta 1 with epidermal growth factor receptor (EGFR) at intercellular adhesion sites. Immunoprecipitation with anti-integrin alpha 2 antibodies or anti-integrin beta 1 antibody resulted in preferential coprecipitation of EGFR from A431 cell lysates, while

anti-**EGFR antibody** coprecipitated integrin alpha 2 beta 1. Chemical crosslinking confirmed the association of integrin alpha 2 beta 1 and EGFR. Colocalization of integrin alpha 2 beta 1 and EGFR at cell-cell contact sites was observed by double immunofluorescence staining of **A431 cells**. EGF-induced EGFR stimulation did not affect the association of integrin alpha 2 beta 1 and EGFR. However, immunostaining with the antibody specific to activated-EGFR revealed that EGFR localized at cell-cell contact sites are phosphorylated even in serum-depleted conditions, while EGFR localized to other sites is totally dephosphorylated in the same conditions. The EGFR phosphorylation in cell-cell contact sites observed in a serum-depleted culture was abrogated with a function-blocking antibody of integrin alpha 2, but not with a non-function-blocking alpha 2 antibody or function-blocking alpha 3 antibody. Moreover, the EGFR phosphorylation in serum-depleted conditions was not observed in suspended cells, or largely abrogated in sparse cells, indicating that cell-cell adhesion is required for EGFR phosphorylation. These results indicate that integrin alpha 2 beta 1 not only physically associates with EGFR but also functions in serum-independent EGFR activation at cell-cell contact sites. The present results shed a new light on the role of intercellular integrins in cell-cell interactions.

L20 ANSWER 5 OF 10 MEDLINE DUPLICATE 4

2000093856 Document Number: 20093856. PubMed ID: 10628369. Epidermal growth factor receptor-dependent cytotoxic effect of anti-**EGFR antibody**-ribonuclease conjugate on human cancer cells. Suwa T; Ueda M; Jinno H; Ozawa S; Kitagawa Y; Ando N; Kitajima M. (Department of Surgery, Keio University School of Medicine, Tokyo, Japan.) ANTICANCER RESEARCH, (1999 Sep-Oct) 19 (5B) 4161-5. Journal code: 59L; 8102988. ISSN: 0250-7005. Pub. country: Greece. Language: English.

AB We have conjugated the murine monoclonal antibody (528) against the human epidermal growth factor receptor (EGFR) to mammalian pancreatic ribonuclease (RNase) via N-succinimidyl-3-(2-pyridyldithio) propionate (SPDP) and 2-iminothiolene (2-IT). The conjugate showed dose-dependent cytotoxicity against EGFR-producing squamous cancer cells (A431, TE8, TE5, Ca9-22) and no detectable cytotoxicity against EGFR-deficient small-cell lung cancer cells (H69). The cytotoxicity of the conjugate was positively correlated with the EGFR numbers of each cell line. The addition of excess 528 antibody to the medium protected **A431 cells** from the conjugate cytotoxicity. This immunoconjugate might be useful for targeted treatment of squamous cell carcinomas hyperexpressing EGFR.

L20 ANSWER 6 OF 10 MEDLINE DUPLICATE 5

1999041949 Document Number: 99041949. PubMed ID: 9822654. Peroxynitrite induces covalent dimerization of epidermal growth factor receptors in A431 epidermoid carcinoma cells. van der Vliet A; Hristova M; Cross C E; Eiserich J P; Goldkorn T. (Center for Comparative Respiratory Biology and Medicine, Department of Internal Medicine, University of California, Davis, California 95616, USA.) JOURNAL OF BIOLOGICAL CHEMISTRY, (1998 Nov 27) 273 (48) 31860-6. Journal code: HIV; 2985121R. ISSN: 0021-9258. Pub. country: United States. Language: English.

AB Irreversible tyrosine modifications by inflammatory oxidants such as peroxynitrite (ONOO-) can affect signal transduction pathways involving tyrosine phosphorylation. The epidermal growth factor receptor (EGFR), a member of the c-ErbB receptor tyrosine kinase family, is involved in regulation of epithelial cell growth and differentiation, and possible modulation of EGFR-dependent signaling by ONOO- was studied. Exposure of epidermoid carcinoma **A431 cells** to 0.1-1.0 mM ONOO-

resulted in tyrosine nitration on EGFR and other proteins but did not significantly affect EGFR tyrosine autophosphorylation. A high molecular mass tyrosine-phosphorylated protein (approximately 340 kDa) was detected in A431 cell lysates after exposure to ONOO-, most likely representing a covalently dimerized form of EGFR, based on immunoprecipitation and/or immunoblotting with alpha-**EGFR antibodies** and co-migration with ligand-induced EGFR dimers cross-linked with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide. Covalent EGFR dimerization by ONOO- probably involved intermolecular dityrosine cross-linking and was enhanced after receptor activation with epidermal growth factor. Furthermore, irreversibly cross-linked EGFR was more extensively tyrosine-phosphorylated compared with the monomeric form, indicating that ONOO- preferentially cross-links activated EGFR. Exposure of **A431 cells** to ONOO- markedly reduced the kinetics of tyrosine phosphorylation of a downstream EGFR substrate, phospholipase C-gamma1, which may be related to covalent alterations in EGFR. Alteration of EGFR signaling by covalent EGFR dimerization by inflammatory oxidants such as ONOO- may affect conditions of increased EGFR activation such as epithelial repair or tumorigenesis.

L20 ANSWER 7 OF 10 MEDLINE DUPLICATE 6

2001287673 Document Number: 21224912. PubMed ID: 11326972. The growth inhibition of anti-EGF receptor monoclonal antibody to human lung adenocarcinoma cells. Chen X; Zhang S; Bai C. (Institute of Respiratory Diseases, Zhongshan Hospital, Shanghai 200032.) CHUNG-HUA CHIEH HO HO HU HSI TSA CHIH CHINESE JOURNAL OF TUBERCULOSIS AND RESPIRATORY DISEASES, (1998 Apr) 21 (4) 233-5. Journal code: CYH; 8712226. ISSN: 1001-0939. Pub. country: China. Language: Chinese.

AB OBJECTIVE: Epidermal growth factor (EGF) and its receptor play a critical role in the growth and regulation of many type of malignant cell. The high level of expression of the EGFR on normal cell lung cancer(NSCLC) and the important role of EGFR in signal transduction make it potentially an excellent target for antibody directed therapy. The purpose of this study was to evaluate the anti-tumor effects of anti-EGFR monoclonal antibody on lung carcinoma cells. METHOD: Preparation of hybrid cell producing anti-EGFR monoclonal antibody using EGF receptors partially purified from **A431 cells** as immunogen. The effect of anti-EGFR monoclonal antibody to human lung cells was investigated in vitro. RESULT: The anti-EGFR monoclonal antibody was shown to inhibit the proliferation of human lung adenocarcinoma cells in vitro, but no growth-inhibitory effect was observed in the human small cell lung cancer cells. The concentration, 15 ug/ml, of anti-**EGFR antibody** could inhibit growth rate by 94% and 88% for human lung adenocarcinoma cell lines SPC A-1 and A549, respectively. CONCLUSION: Our initial results indicate that the anti-EGF receptor monoclonal antibody may be potentially useful for the biotherapy of lung adenocarcinoma.

L20 ANSWER 8 OF 10 MEDLINE DUPLICATE 7

1998155648 Document Number: 98155648. PubMed ID: 9494544. Antitumor activity of anti-epidermal growth factor receptor monoclonal antibodies and cisplatin in ten human head and neck squamous cell carcinoma lines. Hoffmann T; Hafner D; Ballo H; Haas I; Bier H. (Department of Otorhinolaryngology, Heinrich-Heine-University, Dusseldorf, Germany.) ANTICANCER RESEARCH, (1997 Nov-Dec) 17 (6D) 4419-25. Journal code: 59L; 8102988. ISSN: 0250-7005. Pub. country: Greece. Language: English.

AB Head and neck squamous cell carcinomas (HNSCC) frequently display increased levels of epidermal growth factor receptor (EGFR) and since the receptor is located on the cell surface, anti-**EGFR antibodies** appear to be suitable agents for antitumor therapy. We

investigated the effect of murine EMD 55900 and rat ICR 62 monoclonal antibodies (MAb) directed against EGFR both as single agents and in combination with cisplatin. ELISA detection showed the amount of EGFR protein in HNSCC lines UM-SCC-10A, -10B, -11B, -14A, -14B, 14C, -22B and HLac 79, 8029NA, 8029DDP to range between 20 and 8100 fmol/mg protein. Compared to **A431 cells**, seven HNSCC lines were high and three low receptor expressors. Only low levels of TGF alpha were found in the supernatants of some untreated HNSCC lines, probably due to the consumption of TGF alpha by EGFR. Consequently, occupation of EGFR by MAb led to marked accumulation of TGF alpha in cell supernatants. Colorimetric MTT assay showed both MAbs (0.3-30nM) to have comparable dose-dependent growth inhibition which correlated with the EGFR content of the respective cell lines ($p < 0.05$). Using 30nM MAb, seven high receptor expressing HNSCC lines were growth inhibited by at least 20% to a maximum of 61% (mean = 38%). Combined treatment with MAb and cisplatin led to a significant decrease in cisplatin IC50 values in 5 cell lines expressing more than 1200 fmol EGFR/mg (dose modification by factor 2.1-4.1). In conclusion, anti-EGFR MAb exert direct antiproliferative activity in HNSCC lines and show additive effects in combination with cisplatin.

L20 ANSWER 9 OF 10 MEDLINE DUPLICATE 8
1998054129 Document Number: 98054129. PubMed ID: 9393980. Reduced ability of transforming growth factor-alpha to induce EGF receptor heterodimerization and downregulation suggests a mechanism of oncogenic synergy with ErbB2. Gulliford T J; Huang G C; Ouyang X; Epstein R J. (Division of Medicine, Imperial College School of Medicine, Charing Cross Hospital, London, UK.) ONCOGENE, (1997 Oct) 15 (18) 2219-23. Journal code: ONC; 8711562. ISSN: 0950-9232. Pub. country: ENGLAND: United Kingdom. Language: English.

AB The epidermal growth factor receptor (EGFR) is activated by a variety of ligands including EGF and transforming growth factor-alpha (TGFalpha), whereas no ligand for the homologous ErbB2 oncoprotein has yet been identified. Here we use both an ErbB2 phosphoantibody (aPY1222) and an activation-specific **EGFR antibody** to show that low concentrations of EGF induce more efficient tyrosine phosphorylation of ErbB2 in **A431 cells** than does equimolar TGFalpha, while EGFR is more potently activated by TGFalpha. Co-precipitation studies confirm that heterodimerization of activated EGFR and transphosphorylated ErbB2 is readily induced by EGF but not TGFalpha. EGFR downregulation is also more efficiently induced by EGF, suggesting that ligand-dependent modification of ErbB2 may be required to terminate EGFR signalling in cells expressing both receptor types. These findings indicate that EGF and TGFalpha differ in their abilities to induce tyrosine phosphorylation and heterodimerization of ErbB2, and raise the possibility that ErbB2 exerts its oncogenic effect in part by impairing TGFalpha-dependent EGFR downregulation.

L20 ANSWER 10 OF 10 SCISEARCH COPYRIGHT 2001 ISI (R)
93:485347 The Genuine Article (R) Number: LP940. THE GROWTH-RESPONSE OF HUMAN TUMOR-CELL LINES EXPRESSING THE EGF RECEPTOR TO TREATMENT WITH EGF AND OR MABS THAT BLOCK LIGAND-BINDING. MODJTAHEDI H (Reprint); STYLES J; DEAN C. INST CANC RES, HADDOW LABS, IMMUNOL SECT, SUTTON SM2 5NG, SURREY, ENGLAND (Reprint). INTERNATIONAL JOURNAL OF ONCOLOGY (AUG 1993) Vol. 3, No. 2, pp. 237-243. ISSN: 1019-6439. Pub. country: ENGLAND. Language: ENGLISH. *ABSTRACT IS AVAILABLE IN THE ALL AND IALL FORMATS*

AB We have investigated the effect of treatment with epidermal growth factor (EGF) and/or monoclonal antibodies (Mabs) to the epidermal growth factor receptor (EGFR) on the growth in vitro of a number of human tumour cell lines. Mabs ICR10, ICR11, and ICR16 that prevent the binding of both I-125-EGF and I-125-TGFalpha to the EGF receptor were found to inhibit the growth of human tumour cell lines overexpressing the EGF receptor. At high concentrations (5 nM), EGF was also found. to inhibit the growth of HN5, A431 and MDA MB-468 cells whereas the proliferation of SKBR3 and HN6 cells was stimulated. While some of the cell lines (e.g. HN5 and HN6) were very susceptible to growth inhibition by antibodies to the EGFR others, known to secrete autocrine growth factors (e.g. A431 and MDA MB-468), were less affected by these antibodies. Indeed, growth of the latter cell lines was inhibited more effectively by the addition of 5 nM of exogenous EGF than by treatment with 156 nM of the anti-EGFR Mabs ICR16, ICR11, and ICR10. In addition, we show that the growth of HN5 cells, which express very large numbers of EGF receptors (1.4x10(7)/cell), was stimulated at picomolar concentrations of EGF but inhibited at concentrations of EGF in the nanomolar range. Maximal stimulation (25% above control) was observed with the addition of 78 pM EGF to the cultures. The mitogenic effect of low concentrations of EGF and the growth inhibitory effect of nanomolar concentrations of EGF on HN5 cells could be reversed by the addition of 156 nM of the anti-**EGFR antibodies**. We conclude that growth inhibition can follow from either inhibition of signal transduction by blocking ligand binding to the EGFR or from excess binding of ligand. With tumours that produce significant quantities of autocrine growth factors the latter will compete with the Mabs for binding to the receptor and reduce their effectiveness.

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L27 ANSWER 1 OF 3 BIOSIS COPYRIGHT 2001 BIOSIS
2001:377459 Document No.: PREV200100377459. A fully human anti-epidermal growth factor receptor (**EGFr**) **antibody** in patients with renal or prostate cancer. Belldegrun, Arie (1); Pantuck, Allan (1); Figlin, Robert A. (1); Lohner, Michelle E.; **Yang, Xiao-dong**; Roskos, Lorin; Schwab, Gisela; Weiner, Louis M.. (1) Los Angeles, CA USA. Journal of Urology, (May, 2001) Vol. 165, No. 5 Supplement, pp. 182-183. print. Meeting Info.: Annual Meeting of the American Urological Association, Inc. Anaheim, California, USA June 02-07, 2001 ISSN: 0022-5347. Language: English. Summary Language: English.

L27 ANSWER 2 OF 3 BIOSIS COPYRIGHT 2001 BIOSIS
2000:238900 Document No.: PREV200000238900. Inhibition of human cancer growth by ABX-EGF, a fully human anti-EGF receptor monoclonal antibody. **Yang, Xiao-Dong (1)**; **Jia, Xiao-Chi (1)**; Corvalan, Jose R. F. (1); Wang, Ping (1); Wu, Elizabeth (1); Davis, C. Geoffrey (1). (1) Abgenix, Inc, Fremont, CA USA. Proceedings of the American Association for Cancer Research Annual Meeting, (March, 2000) No. 41, pp. 530. Meeting Info.: 91st Annual Meeting of the American Association for Cancer Research. San Francisco, California, USA April 01-05, 2000 ISSN: 0197-016X. Language: English. Summary Language: English.

L27 ANSWER 3 OF 3 MEDLINE DUPLICATE 1
1999194218 Document Number: 99194218. PubMed ID: 10096554. Eradication of established tumors by a fully human monoclonal antibody to the epidermal growth factor receptor without concomitant chemotherapy. **Yang X D** ; **Jia X C**; Corvalan J R; Wang P; Davis C G; Jakobovits A. (Abgenix, Inc., Fremont, California 94555, USA.. yang_xd@abgenix.com) . CANCER RESEARCH, (1999 Mar 15) 59 (6) 1236-43. Journal code: CNF; 2984705R. ISSN: 0008-5472. Pub. country: United States. Language: English.
AB A fully human IgG2kappa monoclonal antibody (MAb), E7.6.3, specific to the human epidermal growth factor (EGF) receptor (EGFr) was generated from human antibody-producing XenoMouse strains engineered to be deficient in mouse antibody production and to contain the majority of the human antibody gene repertoire on megabase-sized fragments from the human heavy and kappa light chain loci. The E7.6.3 MAb exhibits high affinity (KD = 5 x 10(-11) M) to the receptor, blocks completely the binding of both EGF and transforming growth factor alpha (TGF-a) to various EGFr-expressing human carcinoma cell lines, and abolishes EGF-dependent cell activation, including EGFr tyrosine phosphorylation, increased extracellular acidification rate, and cell proliferation. The antibody (0.2 mg i.p. twice a week for 3 weeks) prevents completely the formation of human epidermoid carcinoma A431 xenografts in athymic mice. More importantly, the administration of E7.6.3 without concomitant chemotherapy results in complete eradication of established tumors as large as 1.2 cm3. Tumor eradication of A431 xenografts was achieved in nearly all of the mice treated with total E7.6.3 doses as low as 3 mg, administered over the course of 3 weeks, and a total dose of 0.6 mg led to tumor elimination in 65% of the mice. No tumor recurrence was observed for more than 8 months after the last antibody injection, which further indicated complete tumor cell elimination by the antibody. The potency of E7.6.3 in eradicating well-established tumors without concomitant chemotherapy indicates its potential as a monotherapeutic agent for the treatment of multiple EGFr-expressing human solid tumors, including those for which no effective chemotherapy is available. Being a fully human antibody, E7.6.3 is

expected to exhibit minimal immunogenicity and a longer half-life as compared with mouse or mouse-derivatized MAbs, thus allowing repeated antibody administration, including in immunocompetent patients. These results suggest E7.6.3 as a good candidate for assessing the full therapeutic potential of anti-**EGFr antibody** in the therapy of multiple patient populations with EGFr-expressing solid tumors.

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Minireview

Interactions between the Epidermal Growth Factor Receptor and Type I Protein Kinase A: Biological Significance and Therapeutic Implications[1]

Fortunato Ciardiello[2] and Giampaolo Tortora

Cattedra di Oncologia Medica, Dipartimento di Endocrinologia e Oncologia Molecolare e Clinica, Facoltà di Medicina e Chirurgia, Università di Napoli "Federico II," 80131 Napoli, Italy

Abstract

Peptide growth factors regulate normal cellular proliferation and differentiation through autocrine and paracrine pathways and are involved in cancer development and progression. Among the endogenous growth factors, the epidermal growth factor (EGF)-related proteins play an important role in the pathogenesis of human cancer. In fact, overexpression of EGF-related growth factors such as transforming growth factor α and amphiregulin and/or their specific receptor, the EGF receptor (EGFR), has been detected in several types of human cancers, including breast, lung, and colorectal cancers. Therefore, the blockade of EGFR activation by using anti-EGFR monoclonal antibodies (MAbs) has been proposed as a potential anticancer therapy.

The cAMP-dependent protein kinase (PKA) is an intracellular enzyme with serine-threonine kinase activity that plays a key role in cell growth and differentiation. Two PKA isoforms with identical catalytic (C) subunits but different cAMP-binding regulatory (R) subunits (defined as RI in PKAI and RII in PKAII) have been identified. Predominant expression of PKAII is found in normal nonproliferating tissues and in growth-arrested cells, whereas enhanced levels of PKAI are detected steadily in tumor cells and transiently in normal cells exposed to mitogenic stimuli. Overexpression of PKAI has been correlated recently with poor prognosis in breast cancer patients. Inhibition of PKAI expression and function by specific pharmacological agents such as the selective cAMP analogue 8-chloro-cAMP (8-Cl-cAMP) induces growth inhibition in various human cancer cell lines *in vitro* and *in vivo*.

We have provided experimental evidence of a functional cross-talk between ligand-induced EGFR activation and PKAI expression and function. In fact, PKAI is overexpressed and activated following transforming growth factor α-induced transformation in several rodent and human cell line models. Furthermore, PKAI is involved in the intracellular mitogenic signaling following ligand-induced EGFR activation. We have shown that an interaction between EGFR and PKAI occurs through direct binding of the RI subunit to the Grb2 adaptor protein. In this respect, PKAI seems to function downstream of the EGFR, and experimental evidence suggests that PKAI is acting upstream of the mitogen-activated protein kinase pathway.

We have also demonstrated that the functional interaction between the EGFR and the PKAI pathways could have potential therapeutic implications. In fact, the combined interference with both EGFR and PKAI with specific pharmacological agents, such as anti-EGFR blocking MAbs and cAMP analogues, has a cooperative antiproliferative effect on human cancer cell lines *in vitro* and *in vivo*. The antitumor activity of this combination could be explored in a clinical setting because both the 8-Cl-cAMP analogue and the anti-EGFR blocking MAb C225 have entered human clinical trial evaluation.

Finally, both MAb C225 and 8-Cl-cAMP are specific inhibitors of intracellular mitogenic signaling that have different mechanisms of action compared with conventional cytotoxic drugs. In this respect, a cooperative growth-inhibitory effect in combination with several chemotherapeutic agents in a large series of human cancer cell lines *in vitro* and *in vivo* has been demonstrated for anti-EGFR blocking MAbs or for 8-Cl-cAMP. Therefore, the combination of MAb C225 and 8-Cl-cAMP following chemotherapy could be investigated in cancer patients.

Introduction

Growth factors regulate normal cellular proliferation and differentiation and are important in initiating and maintaining neoplastic transformation (1). Cancer cells generally exhibit a decreased requirement for exogenous growth factors as compared with normal cells (2). The relaxation in growth factor dependency is due in part to the ability of tumor cells to synthesize growth factors that can regulate their proliferation through autocrine and paracrine mechanisms by activating specific cell membrane receptors (2). Among the endogenously produced peptide growth factors, TGF-α,[3] AR, and CRIPTO are

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[1] Supported by a grant from the Associazione Italiana per la Ricerca sul Cancro.

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[3] The abbreviations used are: TGF, transforming growth factor; AR, amphiregulin; EGF, epidermal growth factor; EGFR, EGF receptor; Grb2, growth factor receptor binding protein 2; SH, Src homology; MAPK, mitogen-activated protein kinase; PKA, cAMP-dependent protein kinase; 8-Cl-cAMP, 8-chloro-cAMP; C, catalytic subunit; R, regulatory subunit; TSH, thyroid-stimulating hormone; MAb, monoclonal antibody; MBO, mixed backbone oligonucleotide.

EGF-related proteins that play an important role in the pathogenesis of several human epithelial cancers (3). TGF-α, AR, and CRIPTO are expressed by the majority of human primary and metastatic breast and colorectal cancers (4–5). Suppression of synthesis of these growth factors by pharmacological tools such as a specific RNA or DNA antisense approach inhibits human colon and breast cancer cell growth (6–9). Both TGF-α and AR bind to and activate the EGFR. Enhanced expression of EGFR has been detected in the majority of glioblastomas, breast, lung, ovarian, colorectal, and renal carcinomas (3). Overexpression of EGFR has been associated with a poor prognosis in several human tumor types, including breast cancer (3). The EGFR is a M_r 170,000 transmembrane glycoprotein with an external binding domain and an intracellular tyrosine kinase domain. Following ligand binding, the EGFR is autophosphorylated on several tyrosine residues in the intracellular domain and dimerizes, creating a series of high-affinity binding sites for various adaptor molecules that are involved in transmitting the mitogenic signaling to the *ras*/MAPK signal transduction pathway (10). In this respect, Grb2 is an adaptor molecule composed of one SH2 domain, which binds to phosphorylated tyrosines on tyrosine kinase receptors, and of two SH3 domains, which bind to proline-rich sequences of signaling proteins such as SOS (11). Grb2 allows the coupling of the activated EGFR to *ras*, phosphatidylinositol kinase, or phospholipase Cγ pathways (11–15). The specific cell membrane receptor for CRIPTO has not yet been identified, although the addition of recombinant CRIPTO protein to human mammary epithelial cells induces the intracellular signaling cascade that leads to MAPK activation (16).

The PKA is an intracellular enzyme with serine-threonine kinase activity that plays a key role in cell growth and differentiation. cAMP acts in mammalian cells by binding to either of two distinct isoforms of PKA, defined PKAI and PKAII. PKAI and PKAII share identical catalytic (C) subunits, but differ in the regulatory (R) subunits (termed RI in PKAI and RII in PKAII, respectively; Ref. 17). The PKA holoenzyme is a tetramer formed of two identical R subunits and two C subunits (17). Upon cAMP binding to the R subunits, the active C subunit is released (17). The synthesis of RI and RII and the relative abundance of PKAI and PKAII isoforms are differentially regulated during differentiation, cell growth, and neoplastic transformation (18). Predominant expression of PKAII is found in normal nonproliferating tissues and in growth-arrested cells, whereas enhanced levels of PKAI are detected steadily in tumor cells and transiently in normal cells exposed to mitogenic stimuli (19). In this respect, PKAI and its regulatory subunit RIα are generally overexpressed in human cancer cell lines and primary tumors and are induced following transformation by certain oncogenes, such as *ras* (18–19). Overexpression of RIα and PKAI has been correlated recently with poor prognosis in breast cancer patients (20). RIα has also been identified in hepatoma × fibroblast cell hybrids as the tissue extinguisher of differentiation (TSE1), an inhibitor of the expression of several genes related to cell differentiation (21). In contrast, constitutive overexpression of PKAII, following infection with a recombinant retrovirus containing the human *RIIβ* gene, induces growth inhibition of human cancer cells, and reverts the transformed phenotype of *ras*-transformed mouse fibroblasts (22–23). Collectively, these data, along with the distinct subcellular location and the differential sensitivity to cAMP concentration for enzyme activation (17–18), suggest that PKAI and PKAII have different functions in the control of cell growth and differentiation.

In recent years, experimental evidence has been provided on a functional link between neoplastic transformation involving the TGF-α-EGFR autocrine pathway and PKAI expression and activity. In this review, we will discuss the biological relevance of the interactions between the activated EGFR and PKAI and we will present data suggesting that the blockade with specific biological agents of the PKAI serine-threonine kinase-dependent pathway and of the EGFR tyrosine kinase-dependent pathway is a potentially useful novel approach in cancer therapy.

The EGFR-PKAI Connection

PKAI Expression Is Linked to TGF-α- and *ras*-dependent Transformation. Several studies have shown an increased expression of RIα and PKAI following transformation by TGF-α or *ras*. On the other hand, an early inhibition of TGF-α and/or *ras* expression is observed after treatment with selective inhibitors of PKAI, such as 8-Cl-cAMP. These studies have suggested a functional involvement of PKAI in the mitogenic signals transmitted through the EGFR and/or p21*ras* pathways. For example, in NRK rat fibroblasts, TGF-α- and *ras*-dependent transformation causes an early induction of RIα and PKAI expression and a parallel reduction of PKAII (24). The selective inhibition of PKAI by 8-Cl-cAMP is followed by inhibition of TGF-α and p21*ras* expression, by increased PKAII expression, and by cell growth arrest (24). In NOG-8 mouse mammary epithelial cells, stable overexpression of TGF-α, following transfection with a recombinant plasmid containing the human TGF-α cDNA, determines neoplastic transformation (25). This event is associated with a fall in RIIβ mRNA expression and a parallel rise of RIα mRNA expression, without major changes in Cα expression (26). Therefore, PKAI becomes the predominant PKA isoform in TGF-α-transformed NOG-8 cells. Treatment of these cells with the specific PKAI inhibitor 8-Cl-cAMP down-regulates PKAI, induces PKAII, and inhibits TGF-α production, restoring the pattern of expression of non-transformed parental cells (26). Similarly to the NOG-8 mouse model, in MCF-10A normal human mammary epithelial cells, overexpression of TGF-α or of an activated *ras* gene causes neoplastic transformation (27). These events are associated with an increase of PKAI expression and a parallel reduction of PKAII. Down-regulation of PKAI by different pharmacological approaches, such as cAMP analogues or antisense oligonucleotides targeted against RIα, inhibits TGF-α expression and induces growth inhibition in MCF-10A cells transformed by either TGF-α or *ras* (28). Further experimental evidence of a functional cross-talk between TGF-α-EGFR-mediated cell transformation and PKAI expression and function has been provided recently in MDA-MB468 human breast carcinoma cells. In MDA-MB468 cells, a TGF-α-EGFR autocrine growth stimulatory pathway is involved in the control of cell growth and transformation (29). The constitutive inhibition of EGFR expression by transfection of MDA-MB468 cells with an antisense

EGFR plasmid vector is accompanied by the selective down-regulation of PKAI expression (30).

PKAI Is Induced by EGFR Activation in Normal Cells. Different studies have disclosed a link between PKA activation and inhibition of EGFR- and/or *ras*-dependent activation of MAPK (31–33), but whether a specific PKA isoform is responsible for this effect has not yet been elucidated. On the other hand, it has been shown that an activated *ras* oncogene inhibits the function of PKA by interfering with the nuclear location of the Cα catalytic subunit, conceivably following release from PKAII (34). Recently, it has been demonstrated that treatment with the cAMP analogue 8-bromo-cAMP or with cAMP-specific phosphodiesterase inhibitors determines inhibition of smooth muscle cell proliferation in rat carotid arteries following injury (35). Because smooth muscle cell proliferation has been shown to be dependent on *ras/raf*-1/MAPK signaling in this model (36), it seems likely that the growth-inhibitory effect is due to the interaction of PKA and *ras*-mediated signals. PKAI has been causally linked to positive regulation of mitogenic signals through the EGFR and *ras* pathways (24, 26, 28). We have shown that PKAI expression is induced in nontransformed MCF-10A human mammary epithelial cells following treatment with EGF or TGF-α, and it is functionally involved in S-phase entry (37). MCF-10A cells possess approximately 250,000 EGFR sites/cell and depend on the presence of EGF or TGF-α in the culture medium for optimal cell growth, because their withdrawal determines growth arrest in the G_0-G_1 phases of the cell cycle. The addition of complete medium containing EGF to quiescent MCF-10A cells induces RIα expression 6–9 h before cells enter S phase (37). Selective down-regulation of PKAI expression by pretreatment with an anti-RIα antisense oligonucleotide blocks S-phase entry of MCF-10A cells following EGF addition, suggesting a role for PKAI in the EGFR-triggered G_1-to-S transition (37). Furthermore, retroviral vector-mediated RIα overexpression enables MCF-10A cells to grow in serum-free medium, bypassing EGF or TGF-α requirement and conferring a phenotype similar to MCF-10A cells transformed by either the *TGF*-α or the *ras* genes (27, 37). Taken together, these data suggest that PKAI mediates the mitogenic signaling by growth factors of the EGF family in human mammary epithelial cells.

It is not yet clear whether PKAI activation is involved only in the downstream propagation of the EGFR-induced mitogenic signaling or whether PKAI is part of the signal transduction cascade induced by other growth factors. In this respect, an early involvement of PKAI following other mitogenic stimuli in different cell types has been reported. In normal human T lymphocytes, CD3 stimulation or phytohemagglutinin addition causes specific PKAI induction and activation within 5–10 min (38–39). In FRTL-5 rat thyroid cells, which depend on TSH for cell proliferation and thyroglobulin synthesis, a rapid induction of RIα mRNA occurs within 30 min after TSH addition with an increase in PKAI that anticipates cell entry into S phase (40). Conversely, inhibition of PKAI synthesis by an anti-RIα antisense oligonucleotide abrogates the TSH-induced mitogenic effect (40). Interestingly, a tumor-specific transforming sequence derived by the fusion of the *ret* tyrosine kinase receptor and the *RIα* genes has been isolated in two human papillary thyroid carcinomas (41).

Direct Interaction of PKAI with the Activated EGFR through Grb2. It is possible that the different biological effects of the two PKA isoforms is due in part to their intracellular localization, which could allow interactions with potentially different adaptor molecules and/or substrates. The subcellular distribution of the PKA isoforms depends also on the interaction with a specific class of anchoring proteins (AKAPs), which may contribute to their functional role (42). PKAII has been found in association with the plasma membrane, the cytoskeleton, the secretory granules, and the nucleus (19, 43–45). PKAI is broadly distributed in the cytoplasm (46) and may also translocate to the cell membrane. In fact, in human T lymphocytes, PKAI is found in the inner face of the cell membrane, where it is associated with the T-cell receptor-CD3 complex after T-cell activation (39, 47). In EGF-stimulated MCF-10A cells, a cell membrane translocation of PKAI anticipates cell entry into S phase (37, 48).

Furthermore, we have shown recently that in MCF-10A both the RIα and Cα subunits, but not the RIIβ subunit, coprecipitate with the ligand-activated EGFR and that they are present in the EGFR macromolecular signaling complex as an activatable PKAI holoenzyme (49). Whole-cell immunofluorescence studies have shown that RIα staining is superimposable to that of EGFR,[4] which, following ligand activation, translocates from the cell membrane to the cytoplasm by endocytosis (15).

PKAI provides a relevant contribution to the propagation of EGFR-activated mitogenic intracellular signaling. In fact, overexpression of PKAI in MCF-10A RIα cells determines a constitutive activation of MAPK, mimicking the effect of EGF addition to quiescent MCF-10A cells (49). In contrast, inhibition of PKAI-mediated signaling by a RIα antisense oligonucleotide or by 8-Cl-cAMP significantly reduces MAPK activation in EGF-stimulated MCF-10A cells (49).

RIα contains a stretch of uncharged amino acids and a NH_2-terminal proline-rich sequence (47), which may potentially bind to SH3 domains (11). We have found that PKAI interacts with the EGFR through RIα binding to either NH_2- or COOH-terminal SH3 domains of Grb2 (49). RIα is associated with Grb2 independently from EGFR activation, suggesting that RIα and Grb2 may form a complex before ligand activation of EGFR and recruitment of Grb2 to autophosphorylated tyrosine residue(s) (49). Because an activatable PKAI holoenzyme is present at the EGFR site following ligand-dependent activation, PKAI may interact with specific substrates involved in the EGFR-dependent signaling cascade. However, it is not yet defined whether PKAI is involved in a specific signaling pathway or participate to the integration of multiple growth factor-induced signals. The identification of PKAI-specific substrates will be an important step to elucidate the role of this PKA isoform in the transduction of mitogenic signals (Fig. 1).

Therapeutic Implications

Inhibition of EGFR. Because experimental and clinical studies have provided evidence for a TGF-α-mediated autocrine

[4] Unpublished results.

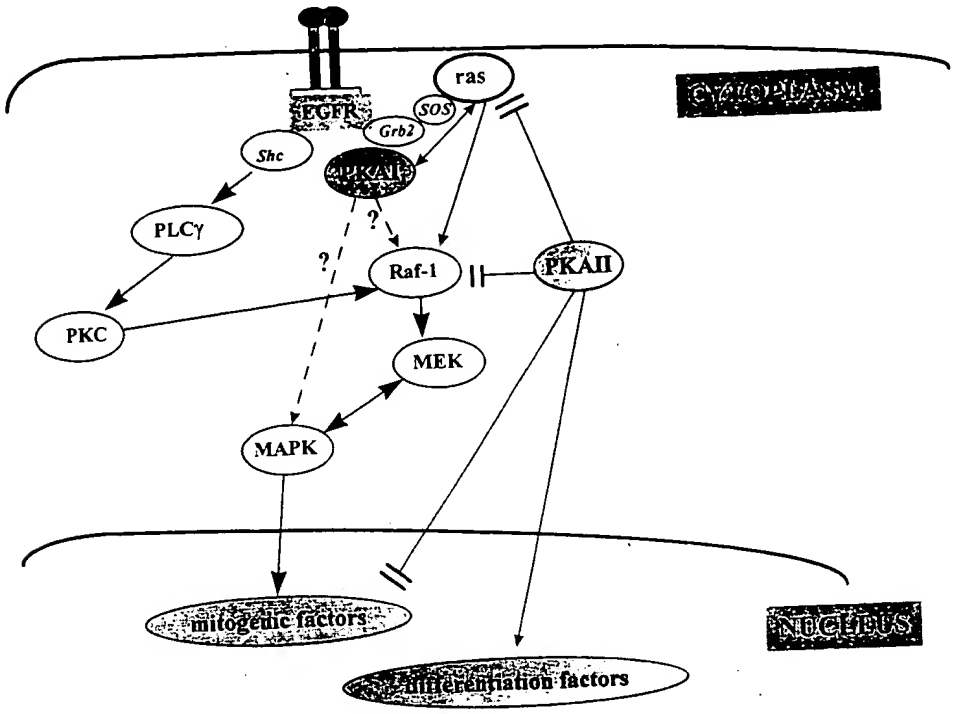

Fig. 1 PKA isoforms in the control of mitogenic signaling. PKAI participates to the EGFR-dependent mitogenic signaling through the binding of the RIα subunit with the SH3 domain of Grb2 adaptor protein recruited at the activated tyrosine receptor site (49). EGFR signaling is not exclusively mediated by PKAI because other pathways are involved, including the phospholipase Cγ/PKC transducing molecules. Inhibition of PKAI markedly reduces MAPK activation (49), but it is unknown whether PKAI directly signals MAPK and *raf*-1. On the other hand, PKAII is able to inhibit PKAI, *ras*, *raf*-1, and, indirectly, MAPK function and expression (18, 31–34). Moreover, PKAII has an inhibitory effect on several nuclear mitogenic factors and a transactivating effect on factors involved with cell differentiation (18, 34). PLCγ, phospholipase Cγ; *MEK*, MAP/extracellular signal-regulated kinase.

growth stimulatory pathway in a variety of human cancers, the blockade of the TGF-α-EGFR autocrine pathway by using anti-EGFR blocking MAbs, recombinant proteins containing TGF-α or EGF fused to toxins, or EGFR-specific tyrosine-kinase inhibitors has been proposed as a potential therapeutic modality (50–57). In this respect, several blocking anti-EGFR MAbs that inhibit the *in vitro* and *in vivo* growth of human cancer cell lines that express TGF-α and EGFR have been generated (58–61). Among these, MAb 528 and MAb 225 are two mouse MAbs that have been extensively characterized for their biological and preclinical properties and represent the first series of anti-EGFR blocking agents that have entered clinical evaluation in cancer patients (50–51, 58–59). MAb 528 and MAb 225 bind to the EGFR with affinity similar to EGF and TGF-α, compete with these ligands for receptor binding, and block EGF- or TGF-α-induced activation of EGFR tyrosine kinase (58–59). In addition, it has been shown that the combined treatment of mice bearing well-established human tumor xenografts with MAb 528 or with MAb 225 and with cytotoxic drugs, such as doxorubicin or cisplatin, significantly increases the antitumor activity of these drugs (62–63). To avoid human anti-mouse antibody production that can interfere with the therapeutic efficacy of repeated administrations of mouse MAbs in humans, a chimeric human-mouse MAb 225 (MAb C225), that contains the human IgG1 constant region, has been developed recently and purified for clinical use and is in early clinical trials in patients with advanced cancer (64–65).

Inhibition of PKAI. The potential usage of cAMP analogues for the therapy of cancer has been widely discussed in the past two decades. However, the lack of selectivity and the high doses required for the available cAMP analogues have been a major obstacle to the development of this approach into feasible clinical trials. A renewed interest has been fostered by the discovery of a new class of site-selective cAMP analogues, which are able to modulate the activity of the PKA at micromolar concentrations (18). 8-Cl-cAMP, the most potent site-selective cAMP analogue, can discriminate between the two cAMP binding sites on RI and RII and is able to down-regulate RIα by facilitating the degradation of the protein, while up-regulating RII expression at the transcriptional level (18, 66). We have shown that down-regulation of RIα by 8-Cl-cAMP is associated with growth inhibition (with IC_{50}s ranging between 0.01 and 10 μM) and differentiation in a wide variety of human cancer cell lines *in vitro* and *in vivo* (66–68). These effects are accompanied by an increased RII:RI ratio and by inhibition *in vitro* and *in vivo* of different oncogenes and growth factor expression, including *ras*, *myc*, *erb*B2, TGF-α, basic fibroblast growth factor, and vascular endothelial growth factor (24, 26, 28, 66–69). The inhibition in the expression of these genes induced by 8-Cl-cAMP treatment is time and dose dependent and occurs at the mRNA level (26, 69). It has been shown recently that 8-Cl-cAMP is able to revert multidrug resistance in a variety of multidrug resistance cancer cell lines, restoring the sensitivity to cytotoxic drugs (70). Although it is still debated whether metabolites such as 8-Cl-adenosine may contribute to the 8-Cl-cAMP effect (19), 8-Cl-cAMP is presently under clinical investigation in Phase II trials, because we have shown in a Phase I clinical trial that 8-Cl-cAMP can be safely administered to cancer patients at doses that achieve plasma concentrations within the potential therapeutic range for growth inhibition (71).

A more direct approach to inhibit the synthesis and function of PKAI has been developed by the use of phosphorothio-

ate-modified antisense oligonucleotides targeted against the 5′ coding sequence of the human RIα mRNA. Treatment with these antisense oligonucleotides suppressed RIα production and determined inhibition of *in vitro* proliferation in various human cancer cell lines and *in vivo* growth of LS-174T human colon cancer xenografts (72–74). Although phosphorothioate-modified antisense oligonucleotides have shown promising results as a first generation of oligonucleotides, a series of novel MBOs targeted against RIα has been generated to further improve their therapeutic potential. MBOs have appropriately placed segments of phosphorothioate oligonucleotides and segments of modified oligodeoxy- or oligoribonucleotides, such as methylphosphonate linkages (75). The anti-RIα second generation antisense oligonucleotides have a significant antiproliferative effect *in vitro* (with IC_{50}s ranging between 0.01 and 1 μM) and *in vivo* in a number of human cancer cell lines.[4] Because MBOs have shown a significant reduction of side effects and a better pharmacokinetic profile *in vivo* as compared with phosphorothioate oligonucleotides (75), they are entering clinical evaluation in cancer patients.

Combined Blockade of EGFR and PKAI. The large body of experimental evidence suggesting a functional link between neoplastic transformation involving TGF-α-induced EGFR activation and PKAI has prompted studies to evaluate whether the double blockade of EGFR and PKAI may have an antiproliferative effect in human cancer cells and may improve the antitumor activity of either blockade alone. In a first series of experiments, we have evaluated the growth-inhibitory effects of the combined treatment with the anti-EGFR MAb 528 and 8-Cl-cAMP on two human colon cancer cell lines (GEO and CBS) and on a human breast cancer cell line (MDA-MB468; Ref. 76). The combination treatment with these two agents had a more than additive growth-inhibitory effect on all three cancer cell lines that secrete TGF-α and express functional EGFRs (76). A 3- to 5-fold reduction in the 8-Cl-cAMP IC_{50} was observed when the tumor cells were exposed to low noninhibitory doses of MAb 528 in combination with 8-Cl-cAMP. Furthermore, treatment with higher concentrations of MAb 528 and 8-Cl-cAMP determined a similar degree of cooperative growth inhibition. We have next demonstrated that the combination of the humanized chimeric anti-EGFR MAb C225 and 8-Cl-cAMP is a highly effective anticancer treatment regimen *in vivo* using human GEO colon carcinoma xenografts as a model (77). The combined blockade of EGFR and of PKAI produced an antitumor effect that is not simply additive. Treatment with low doses of MAb C225 and 8-Cl-cAMP for 5 weeks resulted in a long-term suppression of GEO tumor growth, because tumors resume their growth only after ~8 weeks from cessation of the treatment (77). This effect was accompanied by a statistically significant benefit in animal survival in the group treated with both agents as compared with the groups treated with a single agent. The anticancer effect of the MAb C225 plus 8-Cl-cAMP combination was also accompanied by the suppression in tumor cell production of proteins that function as autocrine growth factors, such as TGF-α, AR, and CRIPTO, or as paracrine angiogenic growth factors, such as vascular endothelial growth factor and basic fibroblast growth factor (77). In addition, a strong inhibition in tumor-induced host neoangiogenesis was observed. The suppression of synthesis of endogenous growth factors has also potential therapeutic relevance. This effect could lead to tumor quiescence in terms of proliferation and neoangiogenic stimulation. Therefore, long-term treatment with anticancer agents that affect intracellular signaling, such as anti-EGFR MAbs and 8-Cl-cAMP, may obtain a control of cancer cell growth and spreading with no toxicity. In fact, MAb C225 and 8-Cl-cAMP treatments were well tolerated by the animals because no signs of toxicity were observed in any treatment group.

We are presently testing whether a similar cooperative antitumor effect could be also obtained by a combination of anti-RIα MBOs and anti-EGFR MAbs. Preliminary experiments in human breast and renal carcinoma cell lines have shown a supradditive growth-inhibitory effect with this approach.[4]

Conclusions

PKAI expression and activation is involved in the intracellular mitogenic signaling following EGFR activation. The interaction between EGFR and PKAI occurs through direct binding of the RIα subunit to the Grb2 adaptor. Therefore, PKAI seems to function downstream to the EGFR, and experimental evidence suggests that PKAI is acting upstream to the MAPK pathway.

The combined interference with EGFR and PKAI with specific pharmacological agents, such as anti-EGFR blocking MAbs and cAMP analogues, has a cooperative antiproliferative effect on human cancer cell lines *in vitro* and *in vivo*. The antitumor activity of this combination could be explored in a clinical setting because both 8-Cl-cAMP and MAb C225 have entered human clinical trial evaluation.

Finally, both anti-EGFR MAbs and 8-Cl-cAMP are inhibitors of intracellular mitogenic signaling with different mechanisms of action compared with cytotoxic agents. In this respect, a cooperative growth-inhibitory effect in combination with several conventional cytotoxic drugs in a large series of human cancer cell lines *in vitro* and *in vivo* has been demonstrated for anti-EGFR blocking MAbs or for 8-Cl-cAMP (62–63, 78). Therefore, the combination of MAb C225 and 8-Cl-cAMP following chemotherapy could be investigated in cancer patients.

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Advances in Brief

Anti-Epidermal Gr wth Factor Recept r M nocl nal Antibody 225 Up-Regulates $p27^{KIP1}$ and Induces G_1 Arrest in Pr static Cancer Cell Line DU145[1]

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Abstract

Autocrine production of transforming growth factor α and overexpression of the epidermal growth factor receptor (EGFR) may contribute to androgen-independent prostatic cancer growth at both primary and metastatic sites. Previously, we showed that human EGFR-blocking monoclonal antibody mAb225 inhibited the growth of DU145 human prostatic cancer cells. Here we explore the hypothesis that mAb225 may act by interfering with cell cycle traversal in these cells. Treatment with mAb225 induced G_1 arrest, which was accompanied by a marked decrease in CDK2-, cyclin A-, and cyclin E-associated histone H1 kinase activities, and a sustained increase in cell cycle inhibitor $p27^{KIP1}$. The increased $p27^{KIP1}$ levels were attributable to elevation of both transcription and translation. CDK2 associated with $p27^{KIP1}$ was increased in mAb225-treated DU145 cells. The retinoblastoma-related protein p130 remained hypophosphorylated in these retinoblastoma-negative cells. These studies demonstrate that the antiproliferative effect of EGFR blockade in DU145 cells may be mediated by up-regulation of $p27^{KIP1}$ at both the mRNA and protein levels.

Introduction

Prostatic cancer, the most prevalent malignancy in North American males, is the second leading cause of cancer death in men (1, 2). Androgen ablation can provide effective palliation. However, in the majority of cases the response is short lived, and proliferation resumes despite castrated levels of androgen. Although the mechanisms are multifactorial, functional autocrine and paracrine growth factor/growth factor receptor interactions are believed to be contributory.

Signaling mediated by receptor tyrosine kinases plays an essential role in the control of cell proliferation. In previous studies, EGF and TGF-α[4] and their receptor (EGFR) have been implicated in the regulation of prostatic cell mitogenesis (3). Human prostatic cancer cell lines derived from hormone-independent cancer express high levels of EGFRs (4, 5). Moreover, coexpression of EGFR and TGF-α has been demonstrated in advanced and metastatic prostatic cancer specimens examined by immunohistochemistry (6). This suggests that a functional autocrine loop may contribute to hormone-independent cancer growth and successful proliferation of prostate cancer at metastatic sites.

We have produced mAbs 225 and 528, which bind to EGFRs with high affinity, block the binding of EGF/TGF-α, and prevent activation of receptor tyrosine kinase (7–10). mAb225 significantly reduced phosphorylation of the EGFR in cultured nontransformed prostatic epithelial cells as well as human prostate carcinoma cell lines PC-3 and DU145, and it inhibited growth of these cells (4, 5).

The capacity of growth factors to regulate cell cycle progression has been characterized as an important aspect of their function. The cell cycle is controlled by the periodic activation of a family of CDKs, which are in turn controlled by interactions with other proteins, including the cyclins and the CDKIs (11). In this study, we define the mechanisms of inhibition of DU145 cell proliferation by EGFR blockade with mAb225. We provide evidence that mAb225 induces G_1 arrest, which is associated with the inhibition of CDK2 activity and induction of the CDKI $p27^{KIP1}$.

Materials and Methods

Cells, Cell Culture, and Cell Proliferation Assays. The DU145 human prostate adenocarcinoma cell line was purchased from the American Type Culture Collection (Bethesda, MD). Cells were grown as monolayers in DMEM with 10% fetal bovine serum. The cell proliferation assay was performed in six-well culture plates in the presence or absence of mAb225. For time points of longer than 3 days, medium was changed every 2 days with re-addition of the antibody. Cells were harvested by trypsinization and counted with a Coulter counter. For flow cytometric analysis of DNA content, cells were cultured with or without mAb225 for various periods and processed for fluorescence-activated cell-sorting analysis as reported previously (12).

CDK Kinase Assay. DU145 cells were treated with mAb225 for indicated time intervals, harvested and washed with PBS by centrifugation, and sonicated. CDK2-, cyclin A-, and cyclin E-associated kinase activities were measured by histone H1 kinase assays as described (12, 13). Briefly, sonicated cell lysates were immunoprecipitated with antibodies against CDK2, CDK4, CDK6, or cyclin A, E, or D (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Washed protein A-Sepharose beads (Repligen Corp., Cambridge, MA) containing immunoprecipitates were resuspended in kinase buffer and [γ-^{32}P]ATP (New England Nuclear) and histone H1 were added. The reactions were stopped after 30 min incubation and analyzed by SDS-PAGE followed by autoradiography. Rb kinase assays were performed on similar preparations as described previously (13), except that a glutathione *S*-transferase-Rb fusion protein (Santa Cruz Biotechnology, Inc.) was used as substrate in the current study.

Western and Northern Blot Analyses. Equal amounts of lysates were used for Western immunoblotting and immunoprecipitation analyses with indicated antibodies as described (12, 13). Briefly, cells were lysed in NP40 lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% NP40, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, and 25 μg/ml of leupetin and aprotinin) and sonicated at 4°C. Proteins from total cell extracts or from immunoprecipitates were separated by SDS-PAGE and blotted with specific antibodies (Santa Cruz Biotechnology, Inc.). For Northern blots, total cellular RNA was extracted by ultracentrifugation of cell lysates in guanidine thiocyanate over cesium chloride cushions. Hybridizations were performed as described (12, 13). The $p27^{KIP1}$ probe was a reverse transcription-PCR-generated human cDNA fragment using a primer pair flanking the whole $p27^{KIP1}$ coding region (primer 1, 5'- ATGTCAAACGTGCGAGTGTC-3'; primer 2, 5'-TTACGTT-TGACGTCTTCTGAG-3').

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[4] The abbreviations used are: EGF, epidermal growth factor; EGFR, EGF receptor; TGF-α, transforming growth factor α; mAb, monoclonal antibody; CDK, cyclin-dependent kinase; CDKI, CDK inhibitor; Rb, retinoblastoma.

Results and Discussion

Effects of mAb225 on DU145 Proliferati n and Cell Cycle Traversal. Initial experiments defined the reduction in DU145 cell proliferation and change in cell cycle phase distribution induced by EGFR blockade with mAb225. As shown in Fig. 1*A*, DU145 proliferation was inhibited by mAb225 treatment. Flow cytometric (fluorescence-activated cell-sorting) analysis indicated that a G_1 arrest was induced by mAb225 in cultured DU145 cells after 24 h treatment (Fig. 1*B*). The increase in the G_1 population was accompanied by a decrease in cells in S phase, whereas the G_2-M population was essentially unchanged.

The Rb protein is one of the key regulators of the G_1-S phase transition (14). Rb and its related proteins, p107 and p130, can bind to various members of the E2F-transcription factor family, preventing their activity. Hyperphosphorylation of Rb, p107, and p130 by CDKs dissociates these proteins from E2F (15). The released E2F may mediate cell cycle progression by activating the genes required for the S phase. Interestingly, DU145 has been demonstrated to be an Rb-deficient cell line (16). Therefore, we investigated the other Rb-related proteins, p107 and p130 (17). As shown in Fig. 1*C*, Western blot analysis indicated that mAb225-treated cells accumulated increased amounts of the hypophosphorylated form of p130, whereas p107 did not show a significant change (data not shown). This suggests that in these Rb-deficient cells, Rb-related protein p130 may be used to regulate cell cycle traversal. Indeed, recent data suggested that phosphorylation of p130 is controlled by the cell cycle machinery and that it may be another key G_1-S phase regulator (18).

Inhibition of CDK2 Activity Is Associated with mAb225-induced Cell Cycle Arrest. Cell cycle traversal is controlled by an interacting set of proteins, including cyclins, CDKs, and CDKIs (11). The transition from G_1 to S phase is thought to be regulated by CDK4 or CDK6 complexed with cyclin D and by CDK2 complexed with cyclins E or A (15). We analyzed the expression and functional status of these molecules in DU145 cells culture with mAb225. CDK2 kinase activity, as measured in CDK2 immunoprecipitates using histone H1 as substrate, was decreased as result of mAb225 treatment of DU145 cells (Fig. 2*A*). The cyclin A- and cyclin E-associated kinase activities also showed a parallel decrease (Fig. 2*A*). These altered activities remained l w for at least 48 h (data not sh wn). In contrast, CDK4- and CDK6-associated kinase and cyclin D1 kinase activities measured using Rb as substrate were not altered significantly after mAb225 treatment (Fig. 2*B*). Except for a slight decrease in D1 protein, mAb225 treatment did not significantly change the amounts of any of these proteins (Fig. 2*C*).

Inhibition of CDK2 Is Associated with Up Regulation of CDKI, p27[KIP1]. The decrease in CDK2-associated kinase activity could not be explained by a change in the levels of CDK2 protein or its cyclin partners. This suggested that mAb225 action on DU145 may activate an additional factor that is responsible for inhibition of CDK2 kinase activity. We therefore investigated whether mAb225 might regulate the levels of expression of the specific inhibitors of CDK2 activity. There are two classes of CDKI that can inhibit CDK activity: the KIP/CIP family, consisting of p21[CIP/WAF1], p27[KIP1], and p57[KIP2], and the INK family consisting of p15[INK4B], p16[INK], p18[INK4C], and p19[INK4D] (13). Western blot analysis of CDKIs showed that p27[KIP1] increased after mAb225 treatment, whereas p21[CIP/WAF1], p15[INK4B], and p19[INK4D] proteins did not change significantly (Fig. 3*A* and data not shown). Next, we analyzed expression of p27[KIP1] and p21[CIP/WAF1] at the mRNA level. Northern blot analysis showed that p27[KIP1] mRNA displayed a moderate increase after addition of mAb225, which could be detected as early as 2 h after treatment (Fig. 3*B*). The p21[CIP/WAF1] mRNA levels did not change after mAb225 treatment (data not shown). Recent data suggest that translational control is primarily responsible for the regulation of p27[KIP1] protein levels under various conditions (19). It also is reported that p27[KIP1] can be regulated through ubiquitin-dependent degradation in serum-starved fibroblasts (20). Our results demonstrate that p27[KIP1] mRNA can be up regulated by the blockade of EGF/EGFR signal transduction pathways. The fact that the increase of p27[KIP1] mRNA preceded the increase in p27[KIP1] protein implies that the up-regulation of mRNA can be at least partially responsible for the increase in p27[KIP1] protein. Thus, the expression of p27[KIP1] protein in DU145 cells may be

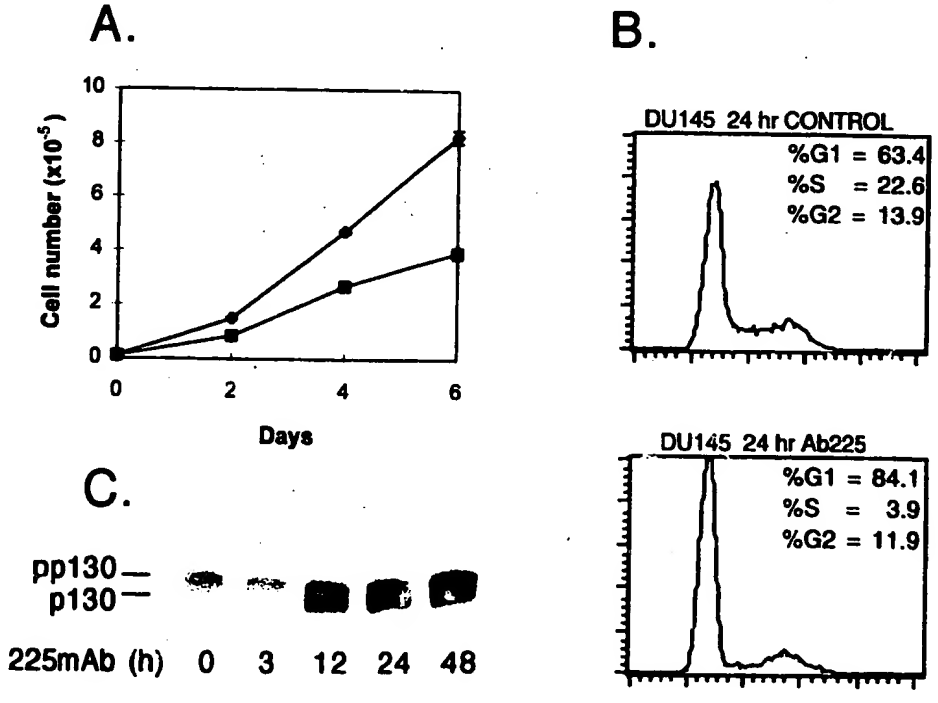

Fig. 1. Cell proliferation, cell cycle distribution and p130 phosphorylation of DU145 cells treated with mAb225. *A*, DU145 cells (1×10^4 cells per well) were grown in six-well culture plates in the presence and absence of 40 nM mAb225. The medium and mAb225 were replaced every two days. Cell numbers were counted with a Coulter Counter at indicated time intervals after treatment. ◆, controls; ■, mAb225 treated. *B*, exponentially growing DU145 cells were treated with mAb225 for 24 hours, and harvested cells were stained with propidium iodide and analyzed for DNA content by flow cytometric analysis. *C*, effect of mAb225 on phosphorylation of the p130 protein. Whole cell lysates from DU145 cells and cells treated with mAb225 for varying time intervals were subjected to electrophoresis for Western blot analysis with an anti-p130 antibody.

regulated at the more usual translational/posttranslational level as well as the mRNA level (Fig. 3, *A* and *B*).

To further investigate whether the up-regulation of $p27^{KIP1}$ contributes to the observed inhibition of CDK2 but not CDK4 and CDK6 activities, we analyzed the physical association of $p27^{KIP1}$ with CDK complexes. Cell lysates of control and mAb225-treated cells were immunoprecipitated with a $p27^{KIP1}$ antibody, and Western blot assays were performed with antibodies against $p27^{KIP1}$, CDK2, and CDK4. As shown in Fig. 3*C*, there was a significant increase of $p27^{KIP1}$-associated CDK2, whereas CDK4 associated with $p27^{KIP1}$ did not change after mAb225 treatment, nor did $p27^{KIP1}$-associated CDK6 (data not shown).

In summary, we have demonstrated that EGFR-blocking antibody mAb225 inhibits proliferation of androgen-independent DU145 prostatic cancer cells by arresting cell cycle progression in G_1. This is mediated by inhibition of CDK2 activity, which is attributable to $p27^{KIP1}$ up-regulation. These changes can explain the marked increase in the amount of hypophosphorylated p130 that we observed in these Rb-negative cells. It is worth noting that in the DiFi human colon adenocarcinoma cell line, which also expresses high levels of EGFR, mAb225 treatment also induced $p27^{KIP1}$ up-regulation and Rb hypophosphorylation with G_1 arrest, followed by apoptosis (13). The DU145 cell line differs from DiFi cells in that it is Rb deficient and the G_1 arrest induced by mAb225 is not followed by apoptosis. Furthermore, the up regulation of $p27^{KIP1}$ mRNA in DU145 cells by treatment with mAb225 is unique. These differences demonstrate the complexity involved in the regulation of cell cycle progression. Further study will attempt to ascertain whether $p27^{KIP1}$ is necessary and sufficient for the G_1 arrest induced by blockade of EGFR-mediated signal transduction pathways with mAb225.

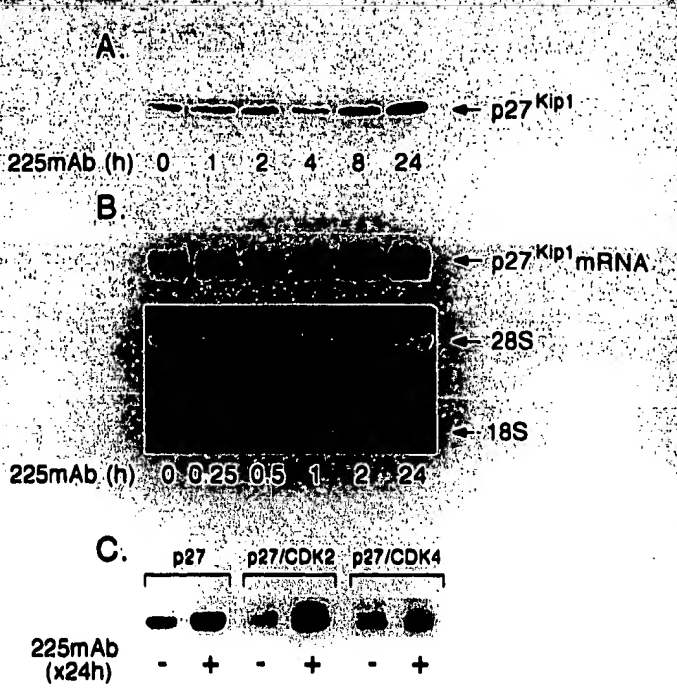

Fig. 3. Induction of $p27^{KIP1}$ protein and mRNA expression by treatment with mAb225. *A*, equal amounts of protein from lysates of control cells and mAb225-treated cells were separated by SDS-PAGE and transferred to filter membranes. Specific antibody was used to detect $p27^{KIP1}$. *B*, samples containing 10 μg total RNA from control and mAb225-treated cells were separated on formaldehyde-agarose gels. RNA was transferred to nitrocellulose filter membranes and probed with a $p27^{KIP1}$ probe (see "Materials and Methods"). *C*, effects of mAb225 on association of $p27^{KIP1}$ with CDK2 and CDK4. Equal amounts of lysates from control and mAb225-treated cells were immunoprecipitated with an anti-$p27^{KIP1}$ antibody. These were then separated by SDS-PAGE and immunoblotted with antibodies against $p27^{KIP1}$, CDK2, or CDK4.

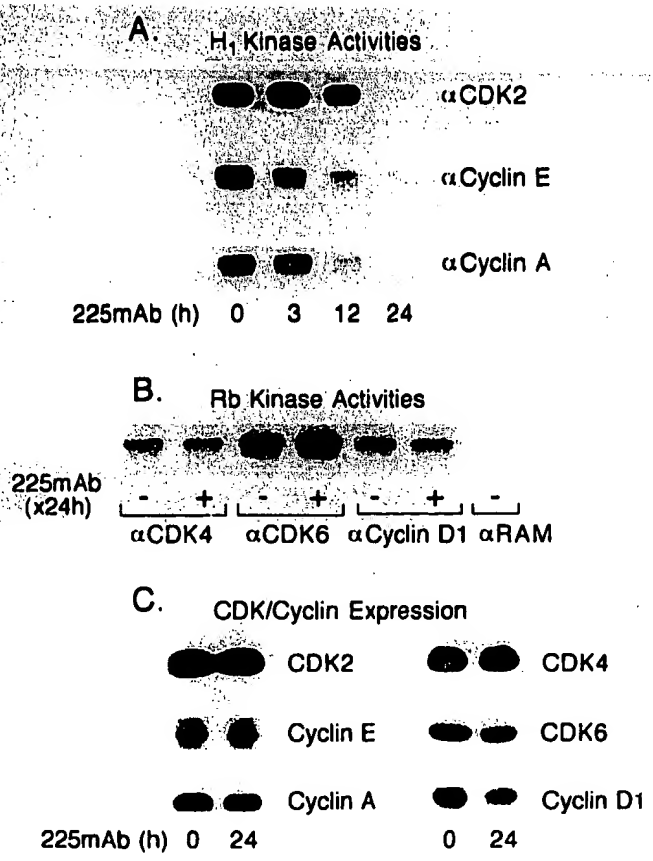

Fig. 2. Effects of mAb225 on G_1 cell cycle CDK kinases. *A*, inhibition of CDK2-, cyclin A-, and cyclin E-associated histone H1 kinase activities with mAb225 treatment. Immunoprecipitates using antibodies against CDK2, cyclin A, or cyclin E were prepared from whole-cell lysates of control and mAb225-treated DU145 cells. Histone H1 kinase assays were carried out with these immunoprecipitates, and histone H1 protein was separated by SDS-PAGE. *B*, effect of mAb225 on *in vitro* CDK4-, CDK6-, and cyclin D1-associated Rb kinase activities. Assays were carried out as in *A*, except that different antibodies were used to immunoprecipitate and a recombinant glutathione *S*-transferase-Rb protein was used as substrate. *C*, effects of mAb225 treatment on the expression of cyclins A, E, and D1 and CDK2, CDK4, and CDK6 proteins. Whole-cell lysates of control and mAb225-treated DU145 cells were separated by SDS-PAGE, and Western blots were probed with antibodies to indicated proteins.

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ANTICANCER RESEARCH *17:* 4419-4426 (1997)

Antitumor Activity of Anti-Epidermal Growth Factor Receptor Monoclonal Antibodies and Cisplatin in Ten Human Head and Neck Squamous Cell Carcinoma Lines

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Abstract. *Head and neck squamous cell carcinomas (HNSCC) frequently display increased levels of epidermal growth factor receptor (EGFR) and since the receptor is located on the cell surface, anti-EGFR antibodies appear to be suitable agents for antitumor therapy. We investigated the effect of murine EMD 55900 and rat ICR 62 monoclonal antibodies (MAb) directed against EGFR both as single agents and in combination with cisplatin. ELISA detection showed the amount of EGFR protein in HNSCC lines UM-SCC-10A, -10B, -11B, -14A, -14B, 14C, -22B and HLac 79, 8029NA, 8029DDP to range between 20 and 8100 fmol/mg protein. Compared to A431 cells, seven HNSCC lines were high and three low receptor expressors. Only low levels of TGF alpha were found in the supernatants of some untreated HNSCC lines, probably due to the consumption of TGF alpha by EGFR. Consequently, occupation of EGFR by MAb led to marked accumulation of TGF alpha in cell supernatants. Colorimetric MTT assay showed both MAbs (0.3-30nM) to have comparable dose-dependent growth inhibition which correlated with the EGFR content of the respective cell lines ($p < 0.05$). Using 30nM MAb, seven high receptor expressing HNSCC lines were growth inhibited by at least 20% to a maximum of 61 % (mean= 38%). Combined treatment with MAb and cisplatin led to a significant decrease in cisplatin IC_{50} values in 5 cell lines expressing more than 1200 fmol EGFR/mg (dose modification by factor 2.1 - 4.1). In conclusion, anti-EGFR MAb exert direct antiproliferative activity in HNSCC lines and show additive effects in combination with cisplatin.*

Epidermal growth factor receptor (EGFR) is a 170 kDa transmembrane phosphoglycoprotein that has been identified in nearly all adult tissues with the exception of hematopoietic cells (1-3). The cysteine-rich extracellular domain of the receptor is responsible for the binding of ligands including epidermal growth factor (EGF), transforming growth factor (TGF) alpha and other polypeptides. Ligand binding causes receptor dimerization and subsequent autophosphorylation, thus initiating a complex intracellular signal transduction pathway which culminates in the phosphorylation of transcription factors and cellular response proteins (3-5). Genetic alterations which affect the regulation of growth factor receptor function or lead to overexpression of the receptor and / or ligand may result in cell proliferation. Overexpression of EGFR has been found in a wide variety of malignancies including head and neck squamous cell carcinomas (HNSCC) (6-14). Since receptor overexpression is often accompanied by increased production of its ligand TGF alpha (15, 16), an autostimulatory pathway has been suggested (17, 18) to play a major role in these malignancies.

Because of the frequency and intensity of EGFR expression, and with regard to its supposed significance in the development and maintenance of the malignant phenotype, EGFR is considered to be a potential target for antitumor therapy in HNSCC. Since the receptor is located at the cell surface membrane, monoclonal anti-EGFR antibodies appear to be suitable agents for the exploration of this approach (19, 20). A number of mouse and rat monoclonal antibodies of different isotypes have been raised against various epitopes, predominantly on the external domain of the human EGFR (21-24). These antibodies were reported to exert several biological activities in a number of in vitro and in vivo models, including modulation of tumor cell proliferation and differentiation, and induction / enhancement of immune effector mechanisms (25-28). Furthermore, interference with receptor-ligand interaction may result in altered tumor cell response to antineoplastic drugs, since growth factors have been shown to provide anti-apoptotic signals (50).

Using ten established cell lines from primary, recurrent and metastatic lesions of HNSCC, we determined the amount of EGFR protein and investigated the effects of two monoclonal anti-EGFR antibodies on a) TGF alpha protein secretion, b) tumor cell proliferation and c) antineoplastic activity of the clinically important drug cisplatin.

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Key Words: Monoclonal antibodies, epidermal growth factor receptor, cisplatin, head and neck cancer.

Materials and Methods

Cell lines and cell culture. Head and neck squamous cell carcinoma lines UM-SCC 10A, 10B, 11B, 14A, 14B, 14C and 22B were kindly provided by T. E. Carey, University of Michigan, Ann Arbor, USA (29, 30). UM-SCC 10A was established from the primary lesion of a larynx carcinoma and 10B from its lymph node metastasis. UM-SCC 14A was derived from a carcinoma of the floor of the mouth, and UM-SCC 14B and 14C represent subsequent local recurrences of the primary tumor. UM-SCC 11B originated from a larynx and UM-SCC 22B from the nodal metastasis of a hypopharynx carcinoma. HLac 79 was established from the lymph node metastasis of a larynx carcinoma (31). Cell line 8029 NA was recloned from HLac 79 by limiting dilution and its subline 8029 DDP has been made resistant to cisplatin by incremental drug exposure (32). The human epidermoid carcinoma line A431 (33, 34) served as a positive control for EGFR protein determination. All cell lines were cultured in plastic culture flasks (Greiner, Solingen, Germany) under standard conditions (37°C, 5% CO_2 in a fully humidified atmosphere) using Minimal Essential Medium (MEM) supplemented with 10% heat-inactivated fetal calf serum (both Gibco, Eggenstein, Germany), 2mM L-glutamine, 50 µg/ml streptomycin and 50 IU/ml penicillin (all ICN, Meckenheim, Germany). For subculturing, cells were detached by exposure to 0.05% trypsin/0.02% EDTA solution (Boehringer, Mannheim, Germany). The study was performed within a maximum of 20 cell passages and all cultures were routinely tested to be free of mycoplasma contamination (DAPI-Test, Boehringer).

Reagents. EMD 55900 (E. Merck, Darmstadt, Germany), originally developed as MAb 425 (23), is a murine IgG 2a antibody produced by hybridoma cells of A431 immunized BALB/c mice. Using breast carcinoma cell line MDA-MB 468 as immunogen IgG 2b rat antibody ICR 62 was generated by H. Modjtahedi *et al* (24). Both antibodies were directed against the external domain of EGFR and have been shown to inhibit ligand binding to EGFR. Stock solutions of 3.42 µM antibody in PBS were prepared, stored at -80°C and diluted in standard culture medium to yield final concentrations of 30, 3 and 0.3 nM.

Cisplatin (cis-diamminedichloroplatinum-II) was stored and prepared according to manufacturer's recommendations (Bristol-Myers-Squibb, Munich, Germany).

EGFR protein assay. EGFR protein was determined with the Human Epidermal Growth Factor Receptor Quantitative ELISA Assay (Oncogene Science, Uniondale, USA), a sandwich type immunoassay using mouse monoclonal capture antibody and biotinylated monoclonal detector antibody specific for the extracellular EGFR domain. The capture antibody which is immobilized on the surface of microtiter plates does not inhibit EGF binding and does not cross react with erbB-2 oncoprotein. Bound antigen is exposed to the detector antibody which is linked to horseradish peroxidase-conjugated streptavidin catalysing the conversion of chromogenic tetra-methylbenzidine. Color intensity was measured with a 96 well multireader spectrophotometer using dual wavelengths of 450/595 nm (Ear 400 ATX, SLT-Labinstruments Crailsheim, Germany). Quantification was achieved by construction of a standard curve using known concentrations of A431 cell lysates. For receptor determination, exponentially proliferating cells in serum supplemented medium were harvested mechanically on day 6 after subculturing, resuspended in a receptor buffer including protease inhibitors (50mM Tris-HCl, containing 5 mM EDTA, 0.5 µg/ml leupeptin, 1 µg/ml pepstatin and 0.2 mM PMSF adjusted to pH 7.4) and lysed with Triton. The soluble cytosolic protein concentration was determined by Bio Rad Protein Microassay (Bio-Rad Laboratories, Munich, Germany). The amount of EGFR/mg protein was determined in three separate experiments using freshly harvested cells. Cell line A431 served as positive control for receptor determination.

TGF alpha assay. TGF alpha protein was determined by a quantitative enzyme-linked immunosorbent assay (Dianova, Hamburg, Germany), utilizing affinity-purified goat polyclonal antibody specific for mammalian TGF alpha. This sandwich ELISA is based on the same test principle as the EGFR assay, and O-phenylenediamin-dihydro-chloride (OPD) served as substrate which was detected at 490 nm after conversion by streptavidin-peroxidase. In three independent experiments, TGF alpha was measured in cell supernatants on day 6 after subculturing of 2×10^5 cells in 6.7 ml/well (6 well plate, Falcon). Medium was replaced on day 3 either as medium containing 30nM anti-EGFR MAb or antibody free medium. In order to detect cell associated TGF alpha, we analysed cell lysates obtained from experiments for EGFR determination. TGF alpha concentrations ranging from 0 to 1000 pg/ml were used to obtain a standard curve. The assay features a minimal detection limit of 10 pg/ml.

In vitro growth inhibition assay. On day 0, exponentionally proliferating cells were harvested from culture flasks, plated in 96-well (6000 cells / 200 ml medium / well) microtiter plates (Becton Dickinson, Heidelberg, Germany) and incubated according to Table I. On day 3, EMD 55900 or ICR 62 were added in different concentrations as single agents or in combination with cisplatin: 0.3-3-30 nM anti-EGFR MAb and 3 cisplatin concentrations were chosen as around the 50% inhibitory drug concentration (IC50) of the cell lines. Cisplatin IC50 values have been determined in previous experiments to be: UM-SCC 10A, 3.4 µg/ml; UM-SCC 10B, 2.6 µg/ml; UM-SCC 11B, 0.76 µg/ml; UM-SCC 14A, 0.44 µg/ml; UM-SCC 14B, 0.52 µg/ml; UM-SCC 14C, 0.78 µg/ml; UM-SCC 22B, 1.1 µg/ml; HLac 79, 0.9 µg/ml; 8029 NA, 1.2 µg/ml; 8029 DDP, 5.8 µg/ml; range: 0.44-5.8 µg/ml (35). Cultures were incubated for another 3 days until controls using medium alone almost reached confluency. The effects of MAb +/- cisplatin were determined by the colorimetric MTT (3-(4,5-dimethylthiazol-2-yl),-2,5-diphenyl-tetrazolium bromide; Sigma, Munich, Germany) assay according to a modification of Carmichael (36). The assay is based on the reduction of a non-toxic water-soluble yellow tetrazolium salt to a purple colored water-insoluble formazan precipitate by the reductive capacity of cytoplasmatic and mitochondrial dehydrogenases present only in living metabolically active cells. On day 6, MTT was added at 5 µg in 50 µl per well for 4 hours. Formazan precipitates were dissolved in 150 µl dimethylsulfoxide (Sigma) and absorbance was measured at 540 nm using a multiscan photometer (Ear 400 ATX). Blanks were wells with all admixtures excluding cells. Optical density / cell survival was calculated according to the formula:

$$\text{fractional absorbance / cell survival} = \frac{\text{absorbance test - absorbance blank}}{\text{absorbance control - absorbance blank}} \times 100$$

MTT assays were performed as sixfold determinations in three independent experiments. Values were analysed using a non-linear estimation procedure (SAS-PC 6.08, SAS Institute Inc., Carey, USA) resulting in sigmoid dose-response curves as described previously (35, 37). The 50% inhibitory drug concentration (IC50) was defined as 50% reduction in absorbance in comparison to untreated controls (100%). The dose-modifying factor (DMF) of 30 nM antiEGFR antibody in combination experiments was calculated as IC50 cisplatin / IC50 (cisplatin + 30nM MAb). To evaluate additive, subadditive or synergistic effects of combined treatment, we established theoretical additive dose response curves according to the method of Poch (38) for analytical comparison with dose response curves obtained from our experiments (logit transformation). Data of MTT assays and ELISAs were correlated. A p-value equal to or smaller than 0.05 was considered to be significant.

Results

EGF receptor assay. In ten HNSCC lines EGFR protein ranged between 20 fmol/mg protein and 8100 fmol/mg protein with a mean of 1956 fmol/mg protein as shown in Table II.

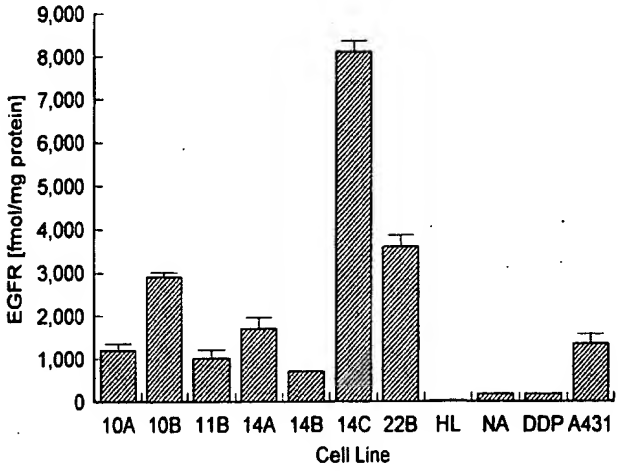

Figure 1. *Epidermal Growth Factor Receptor (EGFR) protein expression in ten squamous cell carcinoma lines of the head and neck. Vertical bars represent standard errors of the mean obtained from three independent experiments.*

Table I. *Time schedule for in vitro growth inhibition assays.*

Day	0	1	2	3	4	5	6
	Cell seed			MEM change			MTT
	Cell seed			MEM change + MAb			MTT
	Cell seed			MEM change + Cisplatin			MTT
	Cell seed			MEM change + Cisplatin + MAb			MTT

Table II. *EGF Receptor and TGF alpha protein in HNSCC lines; minimal detection limit for TGF alpha = 10 pg/ml (n= 3).*

	EGFR [fmol/mg protein]	TGF alpha [pg/ml] in super-natant	TGF alpha [pg/ml] in supernatant +EMD 55900 (30nM)	TGFalpha [pg/ml] in super-natant+ ICR62 (30nM)	TGF alpha [pg/ml] in cell lysate
Cell line					
UM-SCC 10A	1200	–	10	15	–
UM-SCC 10B	2900	10	110	120	160
UM-SCC 11B	1000	10	85	110	–
UM-SCC 14A	1700	–	–	10	–
UM-SCC 14B	700	–	–	10	–
UM-SCC 14C	8100	–	–	–	–
UM-SCC 22B	3600	–	30	35	320
HLac 79	20	–	–	–	–
8029 NA	170	10	75	105	–
8029 DDP4	170	–	–	–	–

UM-SCC 14C exhibited the highest EGFR content in the tumor cell panel and exceeded receptor expression of the classical EGFR overexpressing carcinoma line A431 almost sixfold (Figure 1). Therefore, seven head and neck carcinoma cell lines were designated high EGFR expressors with more than 700 fmol EGFR/mg protein and a mean of 2750 fmol/mg protein: UM-SCC 10A, 10B, 1, 1B, 14A, 14B, 14C, 22B. Three cell lines were low EGFR expressors presenting less than 170 fmol EGFR/mg protein (HLac 79, 8029 NA, 8029 DDP).

TGF alpha assay. Supernatants of only 3 tumor lines showed detectable amounts of 10 pg TGF alpha/ml (Table II). However, incubation with 30 nM anti-EGFR MAb led to markedly elevated TGF alpha levels in culture media of several cell lines. Both mouse EMD 55900 and rat ICR 62 antibody led to a comparable increase of growth factor, which was approximately tenfold for cell line UM-SCC 10B, 11B and 8029 NA (Figure 2). No TGF alpha was detectable in complete culture medium. Determination of cell associated TGF alpha revealed a substantial fraction in two of ten cell lines without correlation to TGF alpha in the supernatant (Table II). No relationship between TGF alpha and EGFR was evident.

In vitro growth inhibition assay. During the 72-hour incubation period, EMD 55900 and ICR 62 produced similar dose-dependent growth inhibition in all HNSCC lines. This antiproliferative effect correlated well with the level of receptor expression ($p<0.05$, Table III). UM-SCC 14C showed the highest EGFR expression among the HNSCC lines tested and appeared to be the most sensitive cell line to antiproliferative MAb activity. All tumor lines with EGFR concentrations exceeding 700 fmol/mg protein were growth inhibited by at least 20%. However, low receptor expressors like HLac 79 and its derivates expressing less than 170 fmol EGFR/mg protein were only poor responders to 30nM MAb. Further increments of antibody concentrations up to 60 and 90nM did not show any additional effect in any cell line (data not shown). Low dose antibodies of 0.3nM showed minor growth stimulation as, for example, shown by UM-SCC 14C cells in Figure 3.

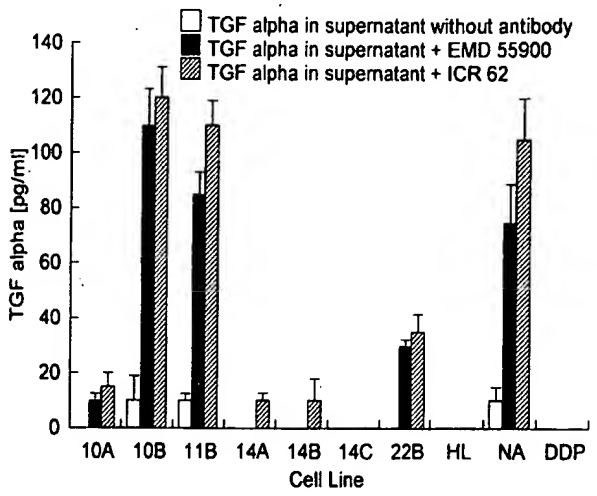

Figure 2. *Transforming Growth Factor (TGF) alpha protein in supernatants of ten squamous cell carcinoma lines of the head and neck. Vertical bars represent standard error of the mean (n= 3).*

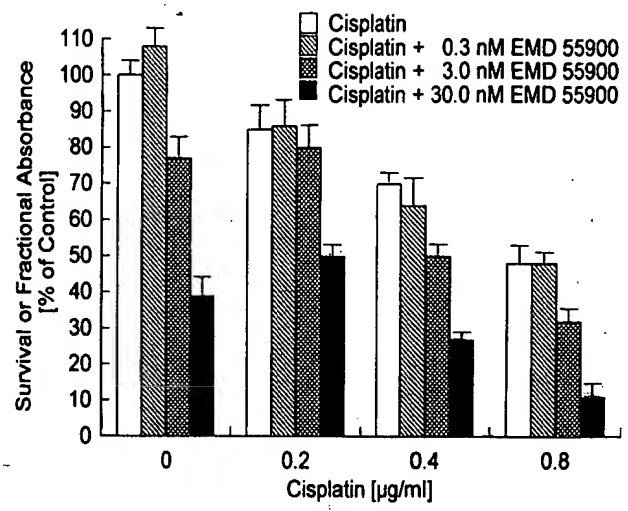

Figure 3. *Dose response curves for cisplatin with different concentration of EDM 55900 in cell line UM-SCC 14C. The data were obtainedfrom sixfold determinations in three independent experiments. Vertical bars represent standard errors of the mean.*

The combination of high dose antibody with cisplatin led to additional growth inhibition demonstrated by a significant decrease of cisplatin IC50 values (Figure 4). Combined treatment with 0.3-30 nM MAb and 0.2-0.4 µg/ml cisplatin is shown by UM-SCC 14C cells as an example in Figure 3. Using 30nM Mab, DMF varied from 0.9 - 4.1 for all cell lines and ranged between 2.1 and 4.1 for 5 cell lines expressing more than 1200 fmol/mg EGFR (Table III). In four of the five tumor lines increased growth inhibition was due to an additive effect of MAb and cisplatin, and only in case of UM-SCC 22B cells there was evidence for synergistic interaction.

Table III. *EGF Receptor status, Growth inhibition by MAb and Dose Modifying Factor (IC50 Cisplatin /IC50 Cisplatin + MAb) of MAb (n= 3).*

	EGFR [fmol/ mg protein]	Growth inhibition [%] EMD 55900 (30nM)	Growth inhibition [%] ICR 62 ICR 62 (30nM)	DMF EMD 55900 (30nM)	DMF ICR 62 (30nM)
Cell line					
UM-SCC 10A	1200	55	48	2.8	2.1
UM-SCC 10B	2900	60	53	4.1	3
UM-SCC 11B	1000	25	25	1.3	1.1
UM-SCC 14A	1700	34	20	3	2.1
UM-SCC 14B	700	27	20	1.3	1.5
UM-SCC 14C	8100	61	55	3.6	3
UM-SCC 22B	3600	22	22	2.5	2.3
HLac 79	20	15	14	0.9	0.9
8029 NA	170	13	10	0.9	0.9
8029 DDP4	170	11	10	1	1.2

Discussion

Increased EGFR expression has been described for several tumor types, including HNSCC. Compared to the classical EGFR overexpressing carcinoma line A 431 (33, 34), seven of ten HNSCC lines tested in the present study exhibited high EGFR levels. Several investigators have looked at the DNA, RNA and protein level of EGFR expression in HNSCC. Most groups applied immunocytochemical techniques or a competitive binding assay to detect EGFR protein and found increased receptor levels to be a constant feature of HNSCC *in vitro* (6, 7). However, two papers have questioned the significance of EGFR overexpression in vitro. In a series of eight oral carcinoma cell lines, Prime *et al* (39) were able to detect only one population that overexpressed EGFR, while the remainder expressed receptor numbers similar to non-malignant oral keratinocytes. Stanton *et al* (40) found elevated EGFR levels in eight of ten HNSCC lines, but only two of the original tumors overexpressed EGFR when compared with normal tissue. The corresponding nude mouse xenografts displayed receptor levels as did the primary tumors, even though they were derived from cell lines that significantly overexpressed EGFR. The authors concluded that HNSCC have a latent potential to overexpress EGFR under *in vitro* culture conditions. On the other hand, the majority of immunohistochemical investigations in freshly

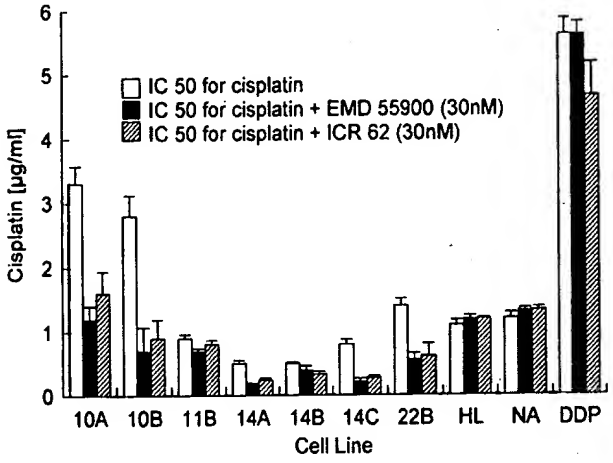

Figure 4. *The 50% inhibitory drug concentration for cisplatin alone and in combination with 30 nM EMD 55900 or ICR 62 in ten head and neck squamous cell carcinoma lines. Vertical bars represent the standard error of the mean obtainedfrom sixfold determinations in three independent experiments.*

resected HNSCC tumors revealed a strong receptor expression, and the intensity of immunostaining was judged to be equal or superior to the reaction that was found in the basal layer of normal epithelium (8, 9, 11-13). Therefore, preclinical investigations on EGFR performed in established cell lines may sufficiently reflect the *in vivo* situation, but *in vitro* artefacts have to be taken into consideration.

In HNSCC, EGFR overexpression has been described to be accompanied by an increased production of TGF alpha suggesting that autocrine and / or paracrine stimulation may be involved in tumor growth. Our experiments in ten HNSCC lines failed to demonstrate a direct correlation between EGFR and TGF alpha production as described elsewhere (15, 16). We detected only low TGF alpha levels in the supernatant of untreated cells, probably due to consumption of TGF alpha by EGFR expressing cells (39, 41). This consumption process, including binding of TGF alpha to the receptor, dimerization and internalization of the receptor-ligand complex occurs within less than one hour (42) and may explain the low TGF alpha levels, which in turn may support the idea of functional active receptors in an autostimulatory pathway. This hypothesis is enhanced by the observed accumulation of TGF alpha in cell supernatants after occupation of EGFR by anti-receptor MAb. Furthermore, a significant amount of TGF alpha appeared to be cell associated in two cell lines. This phenomenon has been described earlier in the renal carcinoma cell line 7860 (43), and transmembrane precursors of TGF alpha have been found to be capable of activating EGFR as well (44, 45).

Using low antibody concentrations (<1nM) Rodeck *et al* (26) have shown growth stimulation in A 431 cells. Similar data were obtained in our experiments, exemplary shown for cell line UM-SCC 14C using 0.3nM EMD 55900. However, antibody concentrations of both 3 and 30 nM produced significant dose-dependent growth inhibition in seven of ten HNSCC lines, with a good correlation to the level of receptor expression ($p<0.05$). The antiproliferative effect observed in 3 HNSCC lines exhibiting low EGFR levels did not exceed 15% of controls and is of doubtful significance. Antibody concentrations exceeding 30nM did not show any additional antiproliferative effect in the cell line panel investigated. In contrast to cisplatin, the establishment of sigmoid dose response curves (35) was not feasible for anti-EGFR MAb. As demonstrated for the high receptor expressor UM-SCC 14C, growth inhibition was limited to a maximum of 61% with 30 nM EMD 55900 and 55% with 30 nM ICR 62, respectively. Such a plateau phase of growth inhibitory effects has been reported for comparable concentrations in carcinoma line A431 using MAb 225 (46).

The exact mechanisms underlying the antitumor activity of anti-EGFR MAbs are still under discussion. In our experiments, TGF alpha accumulation in culture supernatants after antibody application suggests that MAb induced growth inhibition is a consequence of blocking growth factor-receptor interaction. Other cell culture studies have further indicated that this blockade results predominantly in cytostatic rather than cytocidal effects (22, 26). In contrast to natural ligand binding, the blocking antibody does not appear to be internalized but stays at the cell surface for several days (13, 47). Antibody withdrawal has been shown to abrogate MAb induced antiproliferative action within a short time (48), thus supporting the idea of a cytostatic mode of action. On the other hand, there is some evidence that even cytocidal effects may contribute to the antiproliferative activity of anti-EGFR MAbs. For example in the colorectal carcinoma cell line DiFi, programmed cell death was reported after exposure to MAb 225 (49) and it is well established that deprivation of growth factors, *e.g.* TGF alpha, is capable of inducing apoptosis (50). Furthermore, induction of terminal differentiation has been reported in a head and neck carcinoma line with rat ICR 62 (27).

In our studies, combined treatment with anti-EGFR MAb and cisplatin resulted in enhanced antitumor activity in HNSCC lines expressing high EGFR levels. In five cell lines exhibiting a receptor expression of more than 1200 fmol/mg protein, dose-modifying factors>2 were obtained as a result of an additive interaction in four of the five tumor lines. This additive effect is in agreement with data obtained by experiments combining MAb 225 with cisplatin, doxorubicin or paclitaxel (51). Primarily, two independent modes of action should be considered for the anti-EGFR antibody and antineoplastic drug. It may be speculated, however, that the mechanism of enhanced antitumor activity under combined treatment involves a common pathway. DNA is considered to be the critical target molecule of cisplatin, and DNA damage results in either DNA repair or the induction of programmed cell death (52, 53). Since growth factor deprivation may

induce apoptosis as well (49, 50), the interaction between cisplatin chemotherapy and growth factor blockade may augment the course of programmed cell death activation.

Conflicting data have been obtained from investigations combining cisplatin with EGFR ligands (54-57). They showed enhanced sensitivity of tumor cells to cisplatin in combination with EGF instead of anti-EGFR MAb. Activation or inhibition of related signal transduction components, *e.g.* protein kinase C, has been shown to modulate cisplatin sensitivity as well (56, 58). Thus the concept is evolving that signal transduction pathways are involved in the regulation of determinants that are critical for the response of tumor cells to antineoplastic agents, *e.g.* to escape from the cytotoxic insult. Both activation and inhibition of the same signaling step may disturb cellular homeostasis and hereby lead to altered tumor cell response to antineoplastic drugs. This might help to explain why even antagonistic stimuli like anti-EGFR MAbs and receptor ligands are able to enhance chemosensitivity.

Recent clinical studies have provided the initial data on the pharmacokinetics, toxicity and tumor affinity of anti-EGFR MAbs in patients (13, 59). A phase I clinical trial with increasing single doses of EMD 55900 in patients with advanced HNSCC revealed peak serum antibody concentrations of almost 1000nM, exceeding 200nM for more than 3 days without signs of toxicity (13). Good to excellent homogeneous binding of MAb to EGFR was obtained in both primary and metastatic lesions. Thus, MAb concentrations that are required for antineoplastic activty *in vitro* are feasible under *in vivo* conditions.

In conclusion, we were able to demonstrate that murine EMD 55900 and rat ICR 62 anti-EGFR MAb exert similar dose-dependent antiproliferative activity in HNSCC lines correlating with cellular receptor expression. Furthermore, both MAb additively enhanced the effect of cisplatin in HNSCC lines expressing high levels of EGFR.

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Advances in Brief

Cooperative Antiproliferative Effects of 8-Chloro-Cyclic AMP and 528 Anti-Epidermal Growth Factor Receptor Monoclonal Antibody on Human Cancer Cells[1]

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Abstract

8-Chloro-cyclic AMP (8-Cl-cAMP), a site-selective cAMP analogue, is a specific inhibitor of type I cAMP-dependent protein kinase (PKAI) and induces growth inhibition in several human and rodent tumor cell lines. The anti-epidermal growth factor receptor (EGFR) mAb 528 is a blocking antibody able to inhibit the *in vitro* and *in vivo* growth of several human cancer cell lines that express functional EGFRs. Since enhanced levels of PKAI are generally found in tumor cells and an increase in PKAI expression is induced by transformation through a transforming growth factor α/EGFR autocrine pathway, we have evaluated whether treatment with mAb 528 in combination with 8-Cl-cAMP may have an additive or synergistic growth inhibitory effect on human cancer cells. A dose-dependent inhibition of monolayer cell growth was observed in two human colon cancer cell lines (GEO and CBS) and in a human breast cancer cell line (MDA-468) by treatment with either mAb 528 or 8-Cl-cAMP with 50% inhibitory concentration of 2–10 μg/ml or 20–25 μM, respectively. The combined treatment with low noninhibitory doses of mAb 528 (0.25 μg/ml) and with 8-Cl-cAMP had a more than additive growth inhibitory effect with a 3- to 5-fold reduction in the 8-Cl-cAMP 50% inhibitory concentration in all cell lines tested. This combined treatment was similarly effective in inhibiting the soft agar cloning efficiency of GEO cells. 8-Cl-cAMP treatment of GEO cells induced a dose-dependent increase in cell membrane-associated EGFRs with a maximum 3- to 4-fold increase within 48–72 h of treatment. These results suggest that a double blockade of the PKAI serine-threonine kinase-dependent and of the EGFR tyrosine kinase-dependent pathways is potentially useful in cancer therapy.

Introduction

cAMP[3] acts in mammalian cells by binding to either of two distinct isoforms of PKA, defined as PKAI and PKAII (1). PKAI and PKAII share identical catalytic subunits, but differ in the regulatory subunits (termed RI in PKAI and RII in PKAII, respectively; Ref. 1). Differential expression of PKAI and PKAII has been correlated with cell differentiation and neoplastic transformation. In fact, preferential expression of PKAII is found in normal nonproliferating tissues and in growth-arrested cells, while enhanced levels of PKAI are detected in tumor cells and in normal cells following exposure to mitogenic stimuli (2–5). In this respect, PKAI and/or its regulatory subunit RIα are generally overexpressed in human cancer cell lines and primary tumors and are induced following transformation by certain growth factors, such as TGF-α, or oncogenes, such as *ras* and *erb*-B2 (6–9). Furthermore, overexpression of RIα and/or PKAI has been recently shown as a marker of poor prognosis in breast cancer patients (10). For these reasons, PKA has been proposed as a potential target for cancer therapy (2). 8-Cl-cAMP, the most potent of a new class of site-selective cAMP analogues that discriminate between the two cAMP binding sites on RI and RII, is able to down-regulate RIα by facilitating the degradation of the protein while up-regulating at the transcriptional level RII expression (11–14). We have shown that down-regulation of RIα by 8-Cl-cAMP determines growth inhibition and differentiation in a variety of cancer cell lines *in vitro* and *in vivo* (11, 12). These effects are accompanied by an increased RII:RI ratio and by inhibition of different oncogenes and growth factors expression (6–9). Furthermore, we have recently completed a Phase I clinical trial of 8-Cl-cAMP administration in cancer patients refractory to standard therapy.[4]

Experimental and clinical studies have provided evidence for a TGF-α-mediated autocrine growth stimulation pathway in a number of human cancers (15). TGF-α acts by binding to the extracellular domain of the EGFR, thus activating its intracellular tyrosine kinase domain (15). Enhanced expression of TGF-α and/or EGFR has been detected in a majority of glioblastomas, breast, lung, ovarian, colorectal, and renal carcinomas (15). Furthermore, overexpression of EGFR has been as-

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[3] The abbreviations used are: cAMP, cyclic AMP; 8-Cl-cAMP, 8-chloro-cAMP; PKA, cAMP-dependent protein kinase; IC_{50}, 50% inhibitory concentration; EGFR, epidermal growth factor receptor; TGF-α, transforming growth factor α.

[4] G. Tortora, F. Ciardiello, S. Pepe, P. Tagliaferri, A. Ruggiero, C. Bianco, R. Guarrasi, K. Miki, and A. R. Bianco. Phase I clinical study with 8-chloro-cAMP and evaluation of immunological effects in cancer patients. Clinical Cancer Research, in press, 1995.

sociated with a poor prognosis in several human tumor types, such as breast cancer (16). Therefore, the blockade of the TGF-α/EGFR autocrine pathway through the use of specific anti-TGF-α neutralizing or anti-EGFR blocking mAbs has been proposed as a potential therapeutic modality (17, 18). In this respect, several blocking anti-EGFR mAbs that inhibit the *in vitro* and *in vivo* growth of human cancer cell lines have been generated (19–22). mAb 528 is a mouse IgG_{2a} that binds to the EGFR with an affinity similar to that of EGF and TGF-α, competes with these ligands for receptor binding, and blocks EGF-induced or TGF-α-induced activation of EGFR tyrosine kinase (19). mAb 528 inhibits the *in vitro* proliferation of various human tumor cell lines which express TGF-α and EGFR (23). Furthermore, treatment with mAb 528 causes marked tumor growth inhibition in mice given s.c. injections of human cancer cell lines that express high EGFR levels (23, 24). In addition, it has been recently shown that the combined treatment of mice bearing well-established human tumor xenografts with mAb 528 or with mAb 225, a closely related anti-EGFR-blocking antibody, and with cytotoxic drugs, such as doxorubicin or *cis*-diamminedichloroplatinum, significantly increases the antitumor activity of these drugs (25, 26).

Our previous work has suggested a functional link between cell transformation involving a TGF-α/EGFR autocrine pathway and PKAI/RIα expression and activity (7, 8). This observation has prompted us to test whether the blockade of EGFR activation by mAb 528 treatment in combination with down-regulation of RIα by 8-Cl-cAMP treatment may be more effective than each treatment alone in inhibiting the growth of human cancer cell lines that express functional EGFRs. In this report we show that mAb 528 in combination with 8-Cl-cAMP determines a supraadditive growth inhibitory effect on human colon cancer (GEO and CBS) and breast cancer (MDA-468) cell lines.

Materials and Methods

Materials. The biochemical and biological characteristics of mAb 528, a mouse IgG_{2a} mAb that binds to the EGFR with an affinity similar to that of EGF and TGF-α, competes with these ligands for receptor binding, and blocks the EGFR tyrosine kinase activation have been described previously (19, 20). 8-Cl-cAMP, a site-selective cAMP analogue (6), was kindly provided by Dr. K. Miki (Terumo Co., Saitama, Kanagawa, Japan). Mouse ^{125}I-EGF (specific activity, 100–120 μCi/μg) was purchased from Amersham Corp. (Milan, Italy).

Cell Cultures. GEO and CBS cells were kindly provided by Dr. M. Brattain (Baylor College of Medicine, Houston, TX). MDA-468 cells were a gift from Dr. C. Arteaga (Vanderbilt University, Nashville, TN). WIDR cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 20 mM HEPES (pH 7.4), 5 mM glutamine, penicillin (100 units/ml), streptomycin (100 μg/ml; Flow Laboratories, Irvine, Scotland), and insulin (10 μg/ml; Collaborative Biomedical Products, Bedford, MA) in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

Monolayer Growth. To evaluate the effects of 8-Cl-cAMP and/or of mAb 528 on anchorage-dependent cell growth, cells (2×10^3 cells/well) were plated in 96-multiwell cluster dishes (Becton Dickinson, Milan, Italy) and treated every 48 h for three times with the indicated concentrations of 8-Cl-cAMP and/or of mAb 528. Cell growth was evaluated after 7 days using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method, as described previously (27).

Soft Agar Growth. Cells (5×10^3 cells/well) were seeded in 0.5 ml of 0.3% Difco Noble agar (Difco, Detroit, MI) supplemented with complete culture medium. This suspension was layered over 0.5 ml of 0.8% agar-medium base layer in 24-multiwell cluster dishes (Becton Dickinson) and treated with various concentrations of 8-Cl-cAMP and/or of mAb 528. After 12 days the cells were stained with nitroblue tetrazolium (Sigma) and colonies larger than 0.05 mm were counted as described previously (27).

Western Blot Analysis. Protein lysates (50 μg total protein/lane) were separated by SDS-PAGE (4–20% precast gradient gels; Bio-Rad Laboratories, Milano, Italy), transferred to nitrocellulose filters, and incubated with a 1/1000 dilution of an anti-human RIα mouse mAb, as previously described (9).

Flow Cytometric Analysis of Cell Cycle Distribution. Cells were trypsinized, washed twice with Ca^{2+}/Mg^{2+}-free PBS and fixed in 70% ethanol. Cells (10^6) were incubated at room temperature for 30 min in 1 ml of a propidium iodide staining solution (50 μl/ml in Ca^{2+}/Mg^{2+}-free PBS, pH 7.4). DNA analysis was performed in duplicate with a FACScan flow cytometer (Becton Dickinson) coupled with a Hewlett-Packard computer. Cell cycle data analysis was performed by the CELL-FIT program (Becton Dickinson). Pulse area *versus* pulse width gating was performed to avoid doublets from the G_2/M region.

Analysis of DNA Fragmentation. To evaluate the potential induction of programmed cell death by 8-Cl-cAMP and/or by mAb 528, cells were treated every 48 h with various concentrations of 8-Cl-cAMP and/or of mAb 528. After 96 h both adherent and detached cells were harvested, lysed, and DNA was extracted and electrophoresed as described (28).

^{125}I-EGF Binding Assay. The binding assays were performed on cells in monolayer cultures using mouse ^{125}I-EGF as described previously (29). The number of EGF binding sites and the K_d values for specific binding were determined by Scatchard analysis using the EBDA/LIGAND software for fitting multiple binding site data (29).

Results

As illustrated in Fig. 1, treatment with 8-Cl-cAMP induced a dose-dependent inhibition of monolayer growth in human breast (MDA-468) and colon (CBS and GEO) cancer cell lines, with an IC_{50} of 10, 25, and 25 μM, respectively. mAb 528 treatment was also able to induce a dose-dependent growth inhibition under the same culture conditions in all three cancer cell lines, with an IC_{50} of 10, 7, and 2 μg/ml, respectively. To determine whether the combined treatment with mAb 528 and 8-Cl-cAMP may have a cooperative effect on the anchorage-dependent growth, MDA-468, CBS, and GEO cells were treated with a low noninhibitory concentration of anti-EGFR mAb 528 (0.25 μg/ml) and with various concentrations of the cAMP analogue. This combination determined a more than additive antiproliferative effect with a 3- to 5-fold reduction in the 8-Cl-cAMP IC_{50} in all cell lines tested (Fig. 1). We next

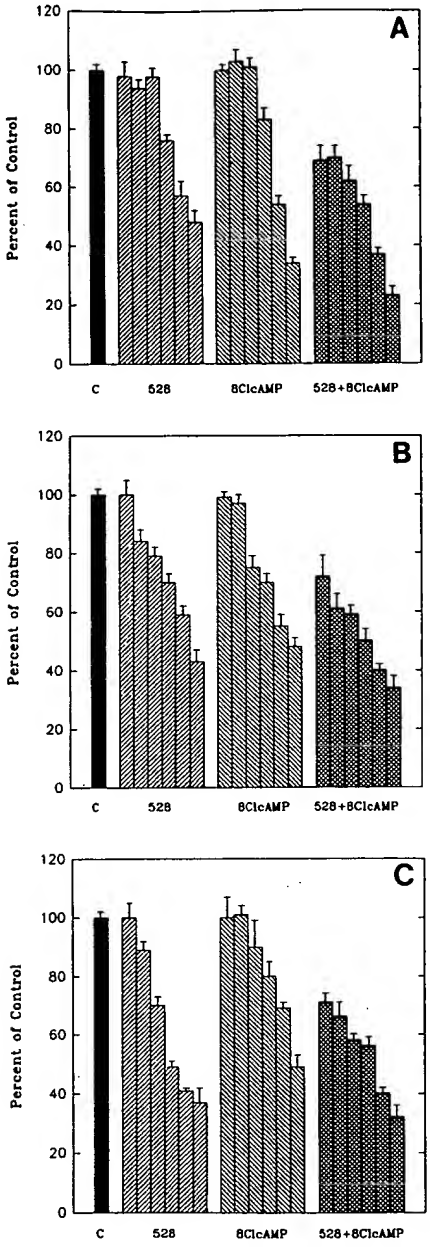

Fig. 1 Effects of mAb 528 and/or 8-Cl-cAMP treatment on anchorage-dependent growth of MDA-468 (*A*), CBS (*B*), and GEO (*C*) human cancer cell lines. Two × 10^3 cells were plated in 96-multiwell cluster dishes and treated every 48 h, for three times with mAb 528 (0.25, 0.5, 1, 2, 5, or 10 μg/ml) or with 8-Cl-cAMP (0.25, 0.5, 1, 5, 10, or 25 μM) or with mAb 528 (0.25 μg/μl) plus 8-Cl-cAMP (0.25, 0.5, 1, 5, 10, or 25 μM). Cell growth was evaluated after 7 days using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method. Results represent the average (±SD) of three separate experiments, each performed in quadruplicate.

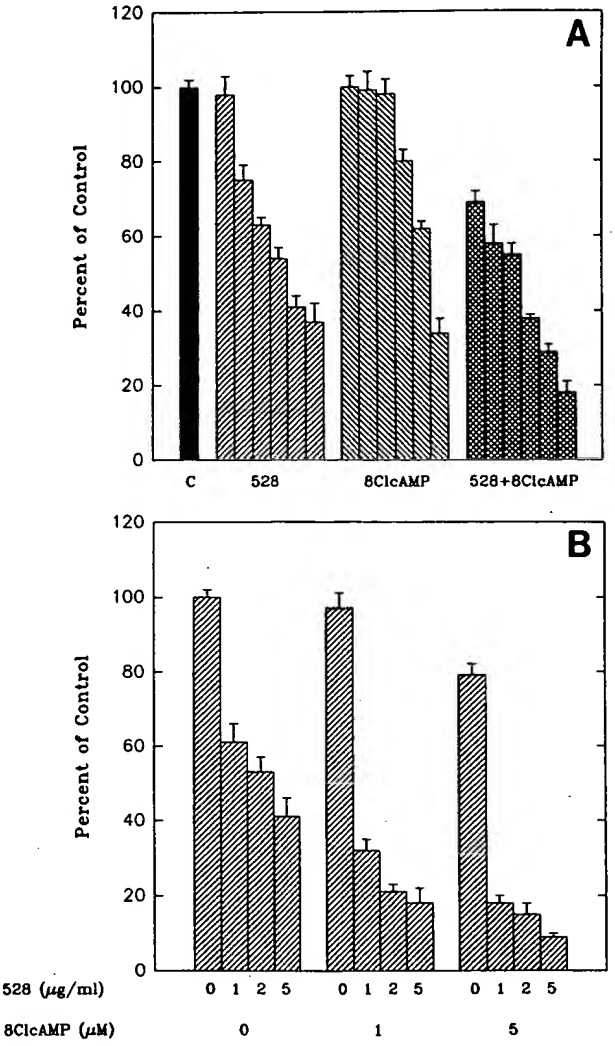

Fig. 2 Effects of mAb 528 and/or 8-Cl-cAMP treatment on anchorage-independent growth of GEO cells. In *A*, 5 × 10^3/well were seeded in 24-multiwell cluster dishes in soft agar and treated with mAb 528 (0.25, 0.5, 1, 2, 5, or 10 μg/ml) or with 8-Cl-cAMP (0.25, 0.5, 1, 5, 10, or 25 μM) or with mAb 528 (0.25 μg/μl) plus 8-Cl-cAMP (0.25, 0.5, 1, 5, 10, or 25 μM). In *B*, cells were seeded in soft agar as above and treated with the indicated concentrations of 8-Cl-cAMP and/or mAb 528. After 12 days colonies larger than 0.05 mm were counted. The growth for untreated controls was 525 colonies/dish (*A*) and 545 colonies/dish (*B*). Results represent the average (±SD) of three separate experiments, each performed in triplicate.

evaluated the effects of mAb 528 and/or 8-Cl-cAMP treatment on the anchorage-independent growth of GEO cells. As shown in Fig. 2*A*, both mAb 528 and 8-Cl-cAMP caused a dose-dependent reduction in soft agar cloning efficiency with an IC_{50} that was similar to that observed in monolayer cultures. Furthermore, treatment with mAb 528 at 0.25 μg/ml in combination with 8-Cl-cAMP lowered the IC_{50} of the cAMP analogue from approximately 18 μM to 3 μM. To ascertain whether the supraadditive growth inhibitory effect of this combination could be obtained also with higher concentrations of mAb 528, GEO cells were treated in soft agar with 1, 2, or 5 μg/ml of the anti-EGFR blocking mAb in combination with 8-Cl-cAMP at 1 or 5 μM. Fig. 2*B* shows that a supraadditive inhibitory effect was obtained also in these conditions. For example, the combination of mAb 528 at 2 μg/ml and 8-Cl-cAMP at 1 μM determined an approximately 80% reduction in soft agar cloning efficiency,

Table 1 Cell cycle distribution of GEO cells following treatment with 8-Cl-cAMP and/or mAb 528[a]

Treatment	Cell cycle distribution (%)		
	G_0-G_1	S	G_2-M
Control	61	32	8
MAb 528 (μg/ml)			
0.25	61	32	8
1	60	33	7
5	54	37	9
10	55	37	8
8-Cl-cAMP (μM)			
1	61	31	9
5	60	32	8
10	61	29	10
25	47	31	22
mAb 528 (0.25 μg/ml) + 8-Cl-cAMP (μM)			
1	59	32	9
5	56	36	8
10	49	35	16
25	42	38	20

[a] 10^5 cells/35-mm dish were plated. Following 4 days of treatment with the indicated concentrations of 8-Cl-cAMP, the cells were trypsinized. Cell cycle distribution was assessed using propidium iodide DNA staining and analyzed with a FACScan flow cytometer. Data represent the average of three separate exeriments. SD was less than 5%.

whereas each treatment alone determined a 45% or a 5% inhibition, respectively.

To evaluate whether 8-Cl-cAMP-induced and/or mAb 528-induced inhibition of GEO cell growth is correlated with a specific perturbation of the cell cycle, cell cycle analysis of GEO cells treated with different concentrations of the two compounds was performed. As compared to control untreated cells, an accumulation of GEO cells in G_2-M was observed with the highest dose (25 μM) of 8-Cl-cAMP (Table 1). These results are in agreement with those of a previous study with human HL-60 leukemia cells (30). In contrast, no gross alteration in cell cycle distribution was detected when GEO cells were treated with the anti-EGFR mAb 528. In addition, the combined treatment of GEO cells with 0.25 μg/ml mAb 528 and with various concentrations of 8-Cl-cAMP did not change the effect of 8-Cl-cAMP on cell cycle distribution (Table 1). Similarly, the combination of higher doses of mAb 528, such as 1 or 5 μg/ml, and of 8-Cl-cAMP determined a perturbation of GEO cell cycle distribution which was comparable to that induced by 8-Cl-cAMP treatment alone (data not shown).

It has been suggested that growth factor deprivation may determine programmed cell death in several cell types (31). Furthermore, it has been recently shown that growth inhibition induced by anti-EGFR-blocking mAb treatment in human DiFi colon cancer cells results in apoptosis (32). Therefore, we evaluated whether a similar phenomenon could occur in GEO cells following treatment with mAb 528 and/or 8-Cl-cAMP. The DNA from control GEO cells and from GEO cells treated for 96 h with mAb 528 (0.25, 1, 5, or 10 μg/ml), with 8-Cl-cAMP (1, 5, 10, or 25 μM), or with both was extracted and analyzed by agarose gel electrophoresis. No presence of chromatin fragmentation into nucleosome ladders was detected (data not shown). Therefore, in our experimental conditions the growth inhibition induced by the anti-EGFR-blocking antibody and/or by the cAMP analogue alone or in combination did not lead to evidence of apoptotic cell death.

We have previously shown that 8-Cl-cAMP treatment specifically induces a down-regulation of the RIα regulatory subunit of PKAI in tumor cells (7–9). To determine whether mAb 528 could interfere with or potentiate this effect, GEO cells were treated for 4 days with different concentrations of 8-Cl-cAMP (1, 5, 10, or 25 μM), with mAb 528 (0.25 μg/ml), or with mAb 528 (1 μg/ml) plus 8-Cl-cAMP (1, 5, or 10 μM). A maximum of 50 to 80% reduction in RIα protein levels was observed using Western blotting in GEO cells treated with 10 or 25 μM 8-Cl-cAMP as compared to control untreated cells (Fig. 3, *Lanes 1–5*). In contrast, mAb 528 treatment alone did not affect RIα protein expression (Fig. 3, *Lane 6*). Furthermore, the combined addition of mAb 528 and 8-Cl-cAMP to GEO cells did not significantly increase the down-regulation of RIα protein levels as compared to GEO cells treated with equivalent concentrations of 8-Cl-cAMP alone (Fig. 3, *Lanes 7–9*).

Several studies have recently demonstrated that treatment of human cancer cells that possess functional EGFRs with cytokines, such as α-interferon, or with cytotoxic drugs, such as 1-β-D-arabinofuranosylcytosine and 5-aza-2′-deoxycytidine, at concentrations able to induce growth inhibition, is accompanied by an up-regulation of EGFR expression on the cell membrane (29, 33, 34). These findings have led to the hypothesis that treatment with pharmacological agents that inhibit tumor cell proliferation while up-regulating growth factor receptor expression may provide a new approach to improve the therapeutic index of antigrowth factor receptor antibodies or immunoconjugates (35). To determine whether 8-Cl-cAMP treatment could interfere with EGFR expression, we examined the specific ^{125}I-EGF binding characteristics on GEO cells. As shown in Table 2, two classes of EGF binding sites with a high and a low affinity were detected by Scatchard analysis on untreated control GEO cells, which possess approximately 40,000 EGFRs/cell. Treatment with 8-Cl-cAMP at a concentration of 5 or 10 μM for 72 h induced a 3- to 4-fold increase in EGF binding sites on GEO cells without any significant change in binding affinities or in the proportion of low and high affinity binding sites. To ascertain the kinetics of this EGFR up-regulation by the cAMP analogue, GEO cells were treated for various periods with different concentrations of 8-Cl-cAMP. A dose-dependent and a time-dependent up-regulation of EGFRs on GEO cells was observed with a maximum 3- to 4-fold increase following 48–72 h of treatment (Fig. 4).

Discussion

There is an extensive body of evidence demonstrating that human cancer cells express different families of growth factors, cytokines, and their cognate receptors, which contribute through intracrine, autocrine, paracrine, and juxtacrine pathways to regulate tumor growth, angiogenesis, and metastasis, and which can be involved in the development of tumor cell resistance to antineoplastic drugs (36). On these bases, efforts have been made to utilize the knowledge at a molecular level of growth factor receptor activation and of the intracellular growth factor-activated signal transduction pathways for the development of

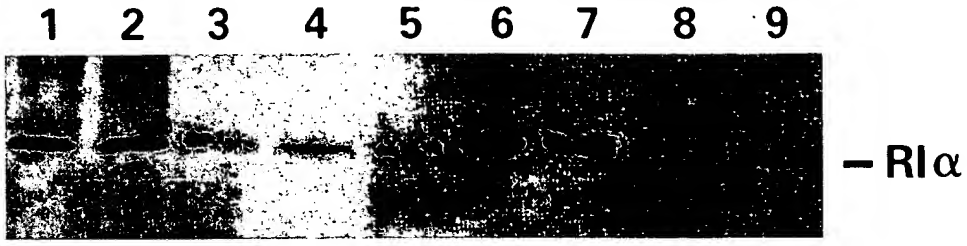

Fig. 3 Western blot analysis of RIα protein expression. *Lane 1*, GEO cells, control; *Lanes 2–5*, GEO cells treated for 4 days with 8-Cl-cAMP (1, 5, 10, or 25 μM, respectively); *Lane 6*, GEO cells treated for 48 h with mAb 528 (0.25 μg/ml), *Lanes 7–9*, GEO cells treated with mAb 528 (0.25 μg/ml) plus 8-Cl-cAMP (1, 5, or 10 μM, respectively).

Table 2 ^{125}I-EGF binding sites on GEO cells[a]

	Sites/cell	
	High affinity (K_d, 0.02 nM)	Low affinity (K_d, 3.4 nM)
Control	6,600	34,000
8-Cl-cAMP (μM):		
1	7,900	40,000
5	25,000	128,000
10	23,500	121,000

[a] Treatment of GEO cells with 8-Cl-cAMP did not result in significant changes of K_d values. Values represent the average (±SD) of two independent experiments, each performed in triplicate. SD was less than 10%.

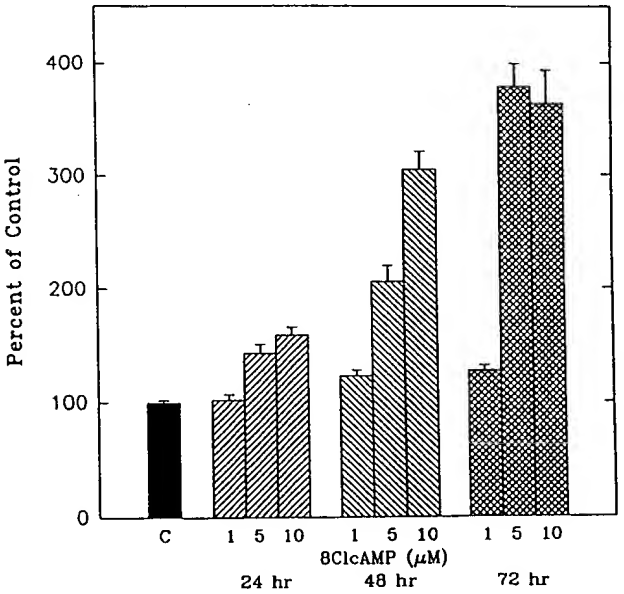

Fig. 4 Percentage of ^{125}I-EGF specifically bound to GEO cells treated with the indicated concentrations of 8-Cl-cAMP for the indicated periods as compared to control nontreated cells. Values represent the average (±SD) of two independent experiments, each performed in triplicate.

new anticancer therapeutic modalities (36). In this respect, interference with the TGF-α/EGFR autocrine pathway is clinically relevant since a TGF-α-mediated autocrine pathway has been demonstrated in several types of human cancers including glioblastomas, breast, lung, ovarian, colorectal, and renal carcinomas (15). For this purpose, several blocking anti-EGFR mAbs that inhibit the *in vitro* and *in vivo* growth of human cancer cell lines have been generated (19–22). On the other hand, site-selective cAMP analogues that cause down-regulation of PKAI and up-regulation of PKAII expression have been demonstrated as effective antineoplastic and differentiating agents (2, 9, 11–14). Since an increase in PKAI expression is observed in human cancer cells in which a TGF-α/EGFR autocrine pathway is operative and there is evidence that PKAI may act downstream to the EGFR (7, 8), we have evaluated whether a double blockade of the EGFR tyrosine kinase activation and the PKAI serine/threonine kinase pathway may have antiproliferative activity in human cancer cells and may improve the anticancer efficacy of either blockade alone. This may represent an important therapeutic goal since both anti-EGFR mAbs and site-selective cAMP analogues have a cytostatic rather than cytocidal activity against most cancer cells *in vitro* (6, 11, 17, 18). Moreover, treatment with anti-EGFR mAbs or with 8-Cl-cAMP causes marked growth inhibition of human tumor xenografts *in vivo*, but does not consistently eliminate well-established xenografts (12, 23, 25, 26).

The results of this study are the first demonstration that the combined interference with the PKAI serine/threonine kinase and with the EGFR tyrosine kinase has a potent antiproliferative activity in human cancer cell lines. In fact, the combined treatment with anti-EGFR mAb 528 and 8-Cl-cAMP had a more than additive growth inhibitory effect on human cancer cell lines that secrete TGF-α and express functional EGFRs. A 3- to 5-fold reduction in the 8-Cl-cAMP IC_{50} was observed when the tumor cells were exposed to low noninhibitory doses of mAb 528 and to 8-Cl-cAMP. Furthermore, treatment of human colon cancer GEO cells with higher concentrations of mAb 528 and 8-Cl-cAMP determined a similar degree of cooperative inhibition of colony formation in soft agar. To elucidate the mechanisms responsible for this cell growth inhibitory effect, we measured the cell cycle distribution and the possible induction of programmed cell death in GEO cells following treatment with mAb 528 and 8-Cl-cAMP alone or in combination. Whereas high doses of 8-Cl-cAMP caused an accumulation of GEO cells in G_2-M, little or no change in cell cycle distribution was observed following treatment of GEO cells with mAb 528. Furthermore, mAb 528 treatment did not alter the 8-Cl-cAMP-induced effect on GEO cell cycle distribution. In addition, the

growth inhibition induced by the anti-EGFR-blocking mAb and/or by the site-selective cAMP analogue did not result in apoptotic cell death of GEO cells.

8-Cl-cAMP-induced cell growth inhibition is accompanied by a specific down-regulation of the RIα regulatory subunit of PKAI in tumor cells (7–9). Therefore, a potential mechanism by which 8-Cl-cAMP and mAb 528 may cooperate could be a more effective reduction in the RIα protein levels. However, this is not the case since mAb 528 treatment alone did not affect RIα protein levels in GEO cells. Furthermore, the combined addition of mAb 528 and 8-Cl-cAMP to GEO cells did not significantly increase the down-regulation in RIα protein expression as compared to GEO cells treated with equivalent concentrations of 8-Cl-cAMP alone.

One of the mechanisms by which 8-Cl-cAMP exerts a supraadditive antiproliferative effect with the anti-EGFR blocking mAb 528 may be the up-regulation of EGFR expression on human cancer cells. In fact, a dose-dependent and a time-dependent up-regulation of EGFRs on GEO cells without any significant change in binding affinities or in the proportion of low and high affinity binding sites was observed with a maximum 3- to 4-fold increase following 48–72 h of treatment. A similar up-regulation in EGFR expression has been observed following treatment of human cancer cells that express functional EGFRs with other antineoplastic agents, such as doxorubicin, α-interferon, 1-β-D-arabinofuranosylcytosine, and 5-aza-2′-deoxycytidine (26, 29, 33, 34). This effect may have potential clinical relevance since it has been proposed that up-regulation of the EGFR on the tumor cell membrane could increase the tumor cell targeting and, therefore, the therapeutic index of anti-EGFR mAbs (35). In this respect, anti-EGFR-blocking mAbs or genetically engineered fusion proteins composed of an EGFR ligand and of a modified bacterial toxin, such as the TGF-α-PE40 *Pseudomonas* esotoxin fusion protein, have been shown to be generally more effective in inhibiting the cell growth of human cancer cell lines that express higher levels of functional EGFR (17, 18, 23, 37).

The results of this study provide the first experimental evidence for a supraadditive antiproliferative effect in human cancer cell lines *in vitro* of two agents that are able to specifically interfere with important steps of the tumor cell signal transduction machinery such as the EGFR tyrosine kinase and the PKAI serine-threonine kinase. We are currently evaluating whether this effect could be obtained also *in vivo* against human tumor xenografts in immunodeficient mice and, therefore, whether the combination of these agents can be tested in cancer patients.

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Antitumor Activity of Combined Blockade of Epidermal Growth Factor Receptor and Protein Kinase A

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Background: **Epidermal growth factor (EGF)-related proteins, such as transforming growth factor-α (TGF-α), control cancer cell growth through hormonal pathways (i.e., autocrine [hormone acts on cell that produces it] and paracrine [hormone acts on nearby cells] pathways). Overexpression of TGF-α and/or its receptor (EGFR) has been detected in human cancers. The blockade of EGFR activation by the use of anti-EGFR monoclonal antibodies (MAbs) has been proposed as a potential anticancer therapy. The type I cyclic adenosine monophosphate (cAMP)-dependent protein kinase (PKAI) is generally overexpressed in human cancer cells and is involved in neoplastic transformation. Inhibition of PKAI by selective cAMP analogues, such as 8-chloro-cAMP (8-Cl-cAMP), induces growth inhibition in various human cancer cell lines.** ***Purpose:*** **On the basis of our previous observations of a cooperative antiproliferative effect of anti-EGFR MAb 528 and 8-Cl-cAMP in human cancer cell lines in vitro, we evaluated the anticancer activity in vivo of the combination of an anti-EGFR MAb (MAb C225) and 8-Cl-cAMP.** ***Methods:*** **Athymic mice were injected subcutaneously with 10^7 human colon carcinoma GEO cells. After 7 days, when established tumor xenografts of 0.30-0.35 cm^3 were detectable, 10-15 mice per group were treated intraperitoneally twice weekly with different doses of 8-Cl-cAMP and/or MAb C225. Cancer cell expression of various growth factors was evaluated by immunohistochemical analysis in tumors obtained from control and treated mice. Data were evaluated for statistical significance using the Student's *t* test and the Mantel–Cox logrank test. All *P* values represent two-sided tests of statistical significance.** ***Results:*** **A 5-week treatment with low doses of 8-Cl-cAMP (0.5 mg/dose) and MAb C225 (0.25 mg/dose) blocked GEO tumor growth (compared with that in control mice; *P*<.00001) and suppressed cancer cell production of autocrine growth factors, such as TGF-α, amphiregulin, and CRIPTO, and of angiogenic (promotes new blood vessel formation) factors, such as vascular endothelial growth factor and basic fibroblast growth factor, with no signs of toxicity. Control and 8-Cl-cAMP (0.5 mg/dose)-treated mice died within 9-10 weeks after tumor cell injection. In MAb C225 (0.25 mg/dose)-treated mice, GEO tumors resumed a growth rate comparable to that in control animals within 3 weeks following the end of treatment and the mice died between 11 and 20 weeks after tumor cell injection. GEO tumor growth was significantly delayed in the MAb C225 plus 8-Cl-cAMP treatment group (*P*<.00001) and was accompanied by a prolonged survival of mice (*P*<.00001) as compared with the control group.** ***Conclusions:*** **Long-term treatment with a combination of agents that selectively inhibit two intracellular signal-transduction enzymes, such as the PKAI serine-threonine kinase and the EGFR tyrosine kinase, has anticancer activity in vivo, reflected by suppression of tumor proliferation and angiogenesis, with no signs of toxicity.** ***Implications:*** **Since these inhibitors of intracellular mitogenic (growth-stimulating) signaling have a different mechanism(s) of action and do not antagonize the effects of cytotoxic therapy, a combination of anti-EGFR MAb C225 and 8-Cl-cAMP should be investigated as a nontoxic, long-term treatment for cancer patients following chemotherapy. [J Natl Cancer Inst 1996; 88:1770-6]**

Epidermal growth factor (EGF)-related growth factors control human cancer growth through autocrine and paracrine mechanisms (*1*). EGF-like growth factors, such as transforming growth factor-α (TGF-α), bind to the extracellular domain of the EGF receptor (EGFR) and activate its intracellular tyrosine kinase domain (*1*). Enhanced expression of TGF-α and/or EGFR has been detected in a majority of human carcinomas (*1*). Overexpression of EGFR has been associated with a poor prognosis in several human tumor types, such as breast cancer (*2*). Therefore, the blockade of the TGF-α/EGFR autocrine pathway by use of anti-EGFR monoclonal antibodies (MAbs) has been suggested as a potential therapeutic modality (*3,4*). Several blocking anti-EGFR MAbs have been generated (*5-7*). MAbs 528 and 225 are two closely related mouse MAbs that bind to the EGFR with affinity similar to EGF and TGF-α, compete with these proteins for receptor binding, and block ligand-induced activation of EGFR tyrosine kinase (*5*). MAbs 528 and 225 have a cytostatic growth-inhibitory effect in vitro and in vivo on human cancer cells that express EGFR (*8*). Treatment of mice bearing established human tumor xenografts with MAb 528 or 225 and with cytotoxic drugs, such as doxorubicin or cisplatin, increases the antitumor activity of these drugs (*9,10*). ^{111}In-labeled mouse MAb 225 has been tested in a phase I trial in patients with advanced cancer (*11*). To avoid human anti-mouse antibody production that can interfere with the therapeutic efficacy of repeated administrations of mouse MAbs in humans, a

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See "Notes" section following "References."

chimeric human–mouse MAb 225 (MAb C225) that contains the human immunoglobulin G1 (IgG1) constant region, has recently been developed and purifed for clinical use (*12*).

Eukaryotic cells possess two distinct cyclic adenosine monophosphate (cAMP)-dependent protein kinase (PKA) isoforms, defined as PKAI and PKAII, that have identical catalytic subunits but differ in the regulatory subunits (termed RI in PKAI and RII in PKAII) (*13*). PKAII is preferentially expressed in nonproliferating cells, while enhanced levels of PKAI are detected in human cancer cells and are induced following transformation by growth factors, such as TGF-α, or oncogenes, such as ras and erbB-2 (*14-17*). We have shown that PKAI is overexpressed in normal cells following exposure to mitogenic stimuli and is involved in S-phase entry (*18-20*). Overexpression of PKAI has been shown as a marker of poor prognosis in breast cancer (*21*). 8-Chloro-cAMP (8-Cl-cAMP) is a site-selective cAMP analogue that specifically inhibits PKAI by facilitating the degradation of the RIα regulatory subunit (*14,22-25*). Decreased expression of RIα by 8-Cl-cAMP induces growth inhibition and differentiation in various cancer cell lines in vitro and in vivo (*22,23*). These effects are accompanied by an increased RII/RI ratio and by the inhibition of different oncogene and growth factor expressions (*15-17*). 8-Cl-cAMP can be safely administered to cancer patients at doses that are able to reach plasma concentrations within the potential therapeutic range for growth inhibition, as we have recently demonstrated in a phase I clinical trial (*26*).

Experimental evidence suggests a functional link between cell transformation involving TGF-α-induced EGFR activation and PKAI/RIα expression and activity (*15,16*). This study was undertaken to evaluate the potential anticancer activity in vivo of the combination of an anti-EGFR MAb and a selective cAMP analogue using the human GEO colon carcinoma cell line grown as a xenograft in nude mice as a model and was based on ur bservations that demonstrated a cooperative antipr liferative effect of anti-EGFR MAb 528 and 8-Cl-cAMP in human cancer cell lines in vitro (*27*).

Materials and Methods

Materials

The biochemical and biologic characteristics of MAb C225, a human–mouse chimeric IgG1 MAb that binds to the EGFR with an affinity fourfold to fivefold higher than the mouse MAb 225, that competes with natural ligands for receptor binding, and that blocks EGFR tyrosine kinase activation, have been previously described (*12*). Clinical grade MAb C225 was provided by N. I. Goldstein, ImClone Systems, New York, NY; clinical grade 8-Cl-cAMP was provided by K. Miki, Terumo Co., Saitama, Kanagawa, Japan. For immunohistochemical evaluation of protein expression in tumor sections, the following antibodies were used. An anti-CRIPTO rabbit antiserum raised against a 17-mer peptide corresponding to amino acid residues 97-113 of the human CRIPTO protein was used at 1:400 dilution (*28*). An anti-amphiregulin (AR) rabbit antiserum generated against a 17-mer peptide corresponding to amino acid residues 159-175 of the rat AR protein was used at 1:200 dilution (*28*). Both the anti-CRIPTO and the anti-AR rabbit antisera were provided by W. J. Gullick, Imperial Cancer Research Fund, London, U.K. An anti-human TGF-α mouse MAb (Ab-2; Oncogene Science, Inc., Manhasset, NY) was used at 1:100 dilution. Each antibody was specific for TGF-α, AR, or CRIPTO and did not cross-react with the other two EGF-related peptides (*28*). An anti-vascular endothelial growth factor H (VEGF) rabbit polyclonal antibody, purchased from Santa Cruz Biotechnology, CA, was used at 1:50 dilution. An anti-basic fibroblast growth factor (bFGF) rabbit polyclonal antibody (Santa Cruz Biotechnology) was used at 1:200 dilution. For proliferation cell nuclear antigen (PCNA) staining, PC 10 MAb (Novocastra Laboratories Ltd., Newcastle upon Tyne, U.K.) was used at 1:200 dilution, as previously reported (*29*). To detect Factor VIII, a rabbit polyclonal antibody (Dakopatts, Glostrup, Denmark) was used at 1:1000 dilution, as previously described (*30*).

GEO Xenografts in Nude Mice

GEO cells, provided by M. Brattain (Baylor College of Medicine, Houston, TX), were maintained in Dulbecco's modified Eagle's minimal essential medium, supplemented with 10% heat-inactivated fetal bovine serum, 20 m*M* HEPES (pH 7.4), 5 m*M* glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin (ICN Biochemicals, Inc., Irvine, Scotland), and 10 µg/mL insulin (Collaborative Biomedical Products, Bedford, MA) in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. Five- to 6-week-old female BALB/c athymic (nu+/nu+) mice were purchased from Charles River Laboratories, Milan, Italy. The research protocol was approved and mice were maintained in accordance with institutional guidelines of the University of Naples Animal Care and Use Committee. Mice were acclimated to the University of Naples Medical School Animal Facility for 1 week prior to being injected with cancer cells. Mice were given a subcutaneous injection of 10^7 GEO cells that had been resuspended in 200 µL of Matrigel (Collaborative Biomedical Products). After 7 days, when well-established xenografts were detectable with an approximate 0.30-0.35 cm^3 tumor size, mice were treated twice weekly with intraperitoneal injections of different doses of 8-Cl-cAMP or MAb C225 alone or in combination for the indicated period of time. Tumor size was measured using the formula $\pi/6 \times$ larger diameter $\times$ (smaller diameter)^2, as previously reported (*9,10*).

Immunohistochemistry and Evaluation of Immunoperoxidase Staining

Formalin-fixed, paraffin-embedded tissue sections (5 µm) were deparaffinized in xylene and rehydrated in a graded series of ethanol. The slides were then treated for 30 minutes at 20 °C with methanol containing 0.3% hydrogen peroxide to block any endogenous peroxidase activity. After several washes with phosphate-buffered saline (PBS), the sections were blocked for 45 minutes with 10% goat serum, washed with PBS, and incubated overnight with the appropriate primary antibody at 4 °C. Sections were then washed three times with PBS and treated with an appropriate secondary biotinylated goat antibody (1:200 dilution, Vectastain ABC kit; Vector Laboratories, Inc., Burlingame, CA) for 30 minutes, as previously reported (*28-30*). Following several washes with PBS, the slides were reacted for 30 minutes with avidin–biotinylated horseradish peroxidase H complex, rinsed twice in PBS, and incubated for 2 minutes in 0.05% diaminobenzidine and in 0.01% hydrogen peroxide, as previously described (*28-30*). The slides were then rinsed in distilled water, counterstained with hematoxylin, and mounted. Nonspecific staining was evaluated for each specimen using either a similar concentration of preimmune rabbit serum or IgG or by adsorbing the primary antibody with the appropriate immunogenic peptide. Both intensity of staining (– to +++) and percentage of immunopositive cells were scored. Two slides for each sample were evaluated by an investigator (G. Fontanini) blinded to the treatment code. To determine the percentage of positive cells, at least 1000 cancer cells per slide were counted and scored. Specific staining was semiquantitated by assigning a score based on color intensity of the brown diaminobenzidine precipitate (+ = light brown staining; ++ = a moderately brown color; and +++ = an intense brown color), as previously described (*31*).

Statistical Analysis

The Student's *t* test (*32*) and the Mantel–Cox logrank test (*33*) were used to evaluate the statistical significance of the results. All *P* values represent two-sided tests of statistical significance. All analyses were performed with the BMDP New System statistical package version 1.0 for Microsoft Windows (BMDP Statistical Software, Los Angeles, CA).

Results

GEO cells were injected subcutaneously into the dorsal flank of nude mice. After 7 days, when the tumor size was approximately 0.30 cm^3, 10 mice per group were treated intraperitoneally twice

weekly for 3 weeks with different concentrations of either MAb C225 r 8-Cl-cAMP. As shown in Fig. 1, both agents determined a marked tumor growth inhibition. A dose-dependent effect was observed with 8-Cl-cAMP treatment, whereas the three doses of MAb C225 tested were equally effective, with a maximum inhibition of growth observed at day 28 following tumor cell injection. However, with this treatment schedule, GEO tumors resumed a growth rate equivalent to that of controls within 7-10 days after termination of the various treatments (data not shown). We next evaluated the antitumor activity of the combination of MAb C225 and 8-Cl-cAMP on the growth of established GEO xenografts. Starting 7 days after tumor cell injection when the average tumor size was approximately 0.35 cm^3, groups of 15 mice were treated intraperitoneally twice weekly for 5 weeks. 8-Cl-cAMP (0.5 mg/dose) was not able to inhibit tumor growth, whereas MAb C225 (0.25 mg/dose) determined a significant growth inhibition compared with controls (two-sided $P<.00001$) (Fig. 2). Moreover, GEO tumor growth was almost completely abrogated in mice treated with MAb C225 (0.25 mg/dose) plus 8-Cl-cAMP (0.5 mg/dose) and was significantly different as compared with controls (two-sided $P<.00001$) or with MAb C225 (0.25 mg/dose)-treated mice (two-sided $P<.00001$).

EGF-related growth factors such as TGF-α, AR, and CRIPTO regulate GEO cell proliferation and transformation (*31,34*). The majority of cancer cells in the untreated control group expressed high levels of all three growth factors (Table 1). A substantial reduction in TGF-α, AR, and CRIPTO expression was bserved in the MAb C225-treated group, whereas 8-Cl-cAMP, at a dose that did not affect tumor growth, inhibited the expression of TGF-α and AR. The combined treatment with the two agents determined a more marked reduction in b th the percentage of positive GEO cells and the average intensity of staining for all three growth factors and was accompanied by an almost complete suppression of tumor cell proliferation, as assessed by PCNA staining (Table 1).

Cancer cells are able to sustain proliferation, invasion, and metastatic spreading through the production of growth factors, such as VEGF and bFGF, that in a paracrine fashion stimulate endothelial cell proliferation with neoangiogenesis (*35,36*). A reduction in GEO cell expression of both VEGF and bFGF was observed after treatment with 8-Cl-cAMP or with MAb C225 (Table 1). Moreover, VEGF and bFGF production was almost completely suppressed in the group treated with the combination of MAb C225 and 8-Cl-cAMP (Table 1). This effect was accompanied by a strong inhibition in neoangiogenesis, as detected

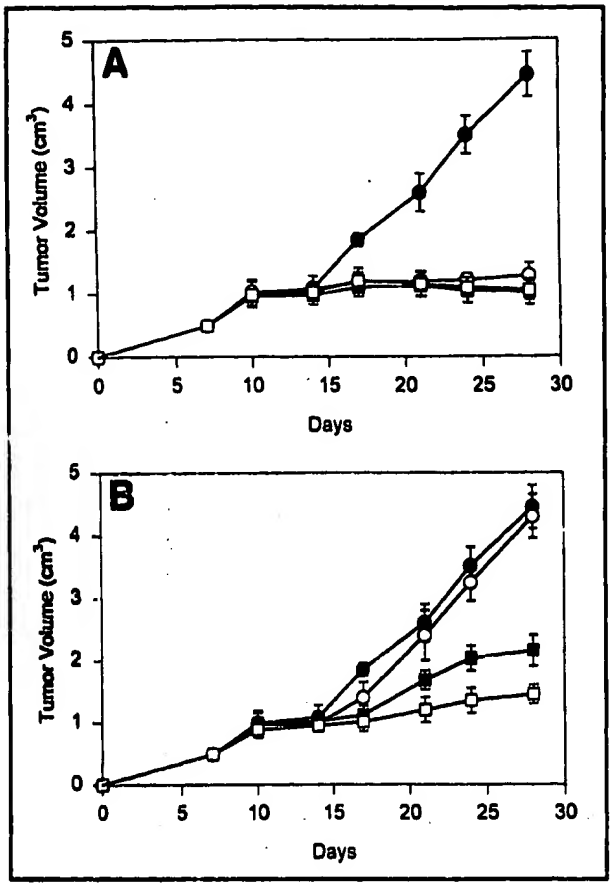


Fig. 1. Antitumor activity of monoclonal antibody (MAb) C225 (A) or 8-chloro-cyclic adenosine monophosphate (8-Cl-cAMP) (B) treatment on established GEO human colon carcinoma xenografts. Mice were injected subcutaneously in the dorsal flank with 10^7 GEO cells as described in the "Materials and Methods" section. After 7 days (average tumor size, 0.30 cm^3), the mice were treated intraperitoneally twice weekly with the indicated doses of either MAb C225 or 8-Cl-cAMP for 3 weeks. Each group consisted of 10 mice. A) control (●); MAb C225: 0.25 mg/dose (○); 0.5 mg/dose (■); and 1 mg/dose (□). B) control (●); 8-Cl-cAMP: 0.5 mg/dose (○); 1 mg/dose (■); and 2 mg/dose (□). Student's *t* test was used to compare tumor sizes among different treatment groups and control untreated mice at day 28 following GEO cell injection. Tumor size in all treatment groups except in the 8-Cl-cAMP (0.5 mg/dose)-treated group was significantly different as compared with controls (two-sided $P<.00001$ for each comparison).

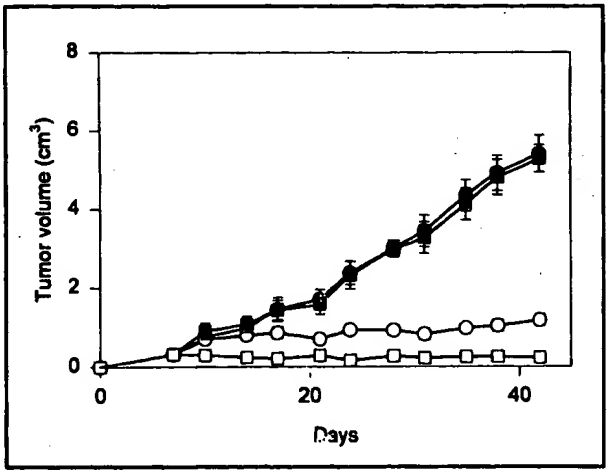


Fig. 2. Antitumor activity of monoclonal antibody (MAb) C225 plus 8-chloro-cyclic adenosine monophosphate (8-Cl-cAMP) treatment on established GEO human colon carcinoma xenografts. Mice were injected subcutaneously in the dorsal flank with 10^7 GEO cells as described in the "Materials and Methods" section. After 7 days (average tumor size, 0.35 cm^3), mice received twice weekly intraperitoneal injections of 8-Cl-cAMP (0.5 mg/dose [■]) or MAb C225 (0.25 mg/dose [○]) or a combination of both agents (□) for 5 weeks. Each group consisted of 15 mice; control untreated mice (●). Student's *t* test was used to compare tumor sizes among different treatment groups at day 42 following GEO cell injection. MAb C225 (0.25 mg/dose) versus control (two-sided $P<.00001$); MAb C225 (0.25 mg/dose) plus 8-Cl-cAMP (0.5 mg/dose) versus control (two-sided $P<.00001$); and MAb C225 (0.25 mg/dose) plus 8-Cl-cAMP (0.5 mg/dose) versus MAb C225 (0.25 mg/dose) (two-sided $P<.00001$).

Table 1. Average tumor size and growth factor expression in GEO human colon carcinoma xenografts*

	Tumor size, cm^3	PCNA, %	Factor VIII
Control	5.4	60	+++
8-Cl-cAMP (0.5 mg/dose)	5.27	60	++
MAb C225 (0.25 mg/dose)	1.17	15	++
8-Cl-cAMP (0.5 mg/dose) + MAb C225 (0.25 mg/dose)	0.23	3	-

	bFGF,%	VEGF,%	AR,%	TGF-α,%	CRIPTO,%
Control	45 (+++)	40 (+++)	60 (+++)	70 (+++)	45 (+++)
8-Cl-cAMP (0.5 mg/dose)	10 (++)	10 (++)	20 (+++)	40 (+++)	40 (+++)
MAb C225 (0.25 mg/dose)	30 (++)	10 (++)	10 (+++)	30 (++)	10 (++)
8-Cl-cAMP (0.5 mg/dose) + MAb C225 (0.25 mg/dose)	2 (+)	4 (+)	5 (+)	15 (+)	5 (+)

*Mice were given a subcutaneous injection of 10^7 GEO cells and treated as described in the legend to Fig. 2. Three mice per group were killed, and tumors were excised on day 42 following GEO cell injection. Immunohistochemistry was performed as described in the "Materials and Methods" section. Percentage of positive tumor cells and average intensity of specific immunostaining were determined as described in the "Materials and Methods" section (+ = light brown color; ++ = moderate brown color; +++ = intense brown color). PCNA = proliferation cell nuclear antigen; 8-Cl-cAMP = 8-chloro-cyclic adenosine monophosphate; MAb = monoclonal antibody; bFGF = basic fibroblast growth factor; VEGF = vascular endothelial growth factor; AR = amphiregulin; and TGF-α = transforming growth factor-α.

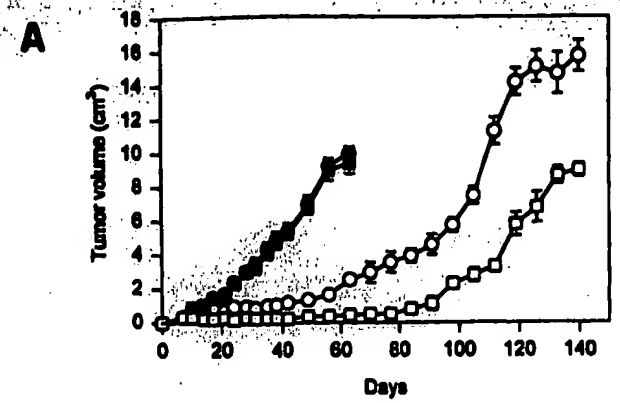
by Factor VIII staining of host blood vessels (Table 1).

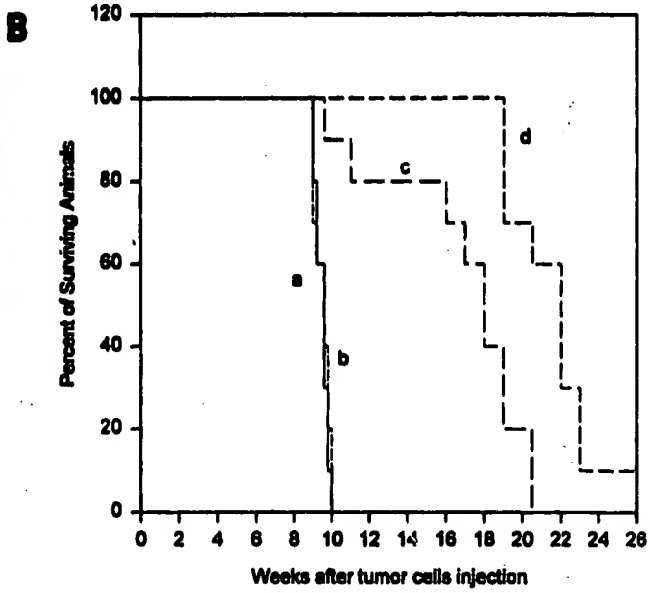
Control untreated mice and 8-Cl-cAMP (0.5 mg/dose)-treated mice died within 9-10 weeks after tumor cell injection (Fig. 3). Tumor growth was significantly slower in MAb C225 (0.25 mg/dose)-treated mice or in the MAb C225 (0.25 mg/dose) plus 8-Cl-cAMP (0.5 mg/dose)-treated group compared with the control group (two-sided $P<.00001$ for both comparisons). In the group of mice treated with MAb C225 alone, GEO tumors resumed a growth rate comparable to controls within 3 weeks following the end of treatment. All of the mice in this group died between 11 and 20 weeks after tumor cell injection. GEO tumor growth was more delayed in the mice that received MAb C225 plus 8-Cl-cAMP treatment. In fact, tumors were quiescent and did not grow for approximately 40 days following the last treatment (Fig. 3). Tumors in this group resumed a growth rate similar to controls only after 55-60 days following the end of treatment. The delayed GEO tumor growth in the MAb C225 plus 8-Cl-cAMP-treated group was accompanied by a prolonged life span of the mice that was significantly different compared with controls (two-sided $P<.00001$) or with the MAb C225 (0.25 mg/dose)-treated group (two-sided $P<.00001$). All of the mice in this group were alive compared with six of 10 animals treated with MAb C225 alone 17 weeks after tumor cell injection (Fig. 3). Most of the mice in the MAb C225 plus 8-Cl-cAMP-treated group died between 21 and 23 weeks after tumor cell injection. One of 10 mice in this group was tumor free from the third week of treatment and was still alive and without evidence of tumor 30 weeks after tumor cell injection. Both MAb C225 and 8-Cl-cAMP alone or in combination were well tolerated by the mice, with no signs of toxicity.

Discussion

Interference with growth factor receptor activation and/or with the intracellular growth factor-activated signal transduction pathways represents a promising strategy for the development of novel anticancer modalities (*37*). In this respect, blocking EGFR function is clinically relevant, since this growth factor receptor is activated in several types of human cancers, including colorectal carcin mas (*1*). An increase in PKAI expression is observed in human cancer cells in which a TGF-α/EGFR autocrine pathway is operative, and there is evidence that PKAI may act downstream to EGFR (*15,16*). Therefore, a combined blockade of the EGFR tyrosine kinase and of the PKAI serine/threonine kinase pathways by interfering with the cancer cell intracellular signaling at two different levels may improve the antitumor activity of a single blockade.

The results of this study demonstrate that the combination of MAb C225 and 8-Cl-cAMP is a highly effective anticancer treatment regimen in vivo using human GEO colon carcinoma xenografts as a model. The combined blockade of EGFR and of PKAI produces an antitumor effect that is not simply additive. Treatment with low doses of MAb C225 and 8-Cl-cAMP for 5 weeks results in long-term suppression of GEO tumor growth, since tumors resume their growth only approximately 8 weeks after cessation f the treatment. This effect is accompanied by a significant benefit in animal survival in the group treated with both agents compared with the groups treated with a single agent. Treatment with MAb C225 plus 8-Cl-cAMP may also result in tumor eradication in a few cases, since one of 10 animals in this group is apparently cured, being alive and tumor free after 30 weeks from tumor cell injection. It is conceivable that a prolonged inhibition of tumor growth, a higher tumor eradication rate, and/or a more significant effect on animal life span can be obtained with a longer treatment protocol. For this purpose, we are currently investigating the effects of a long-term, 10-week treatment period with MAb C225 plus 8-Cl-cAMP. The anticancer effect of the MAb C225 plus 8-Cl-cAMP combination is also accompanied by a suppression in tumor cell production of proteins that may function as autocrine growth factors, such as TGF-α, AR, and CRIPTO, or as paracrine angiogenic growth factors, such as VEGF and bFGF. Although this inhibition may be only a reflection of the impaired growth of GEO tumors, there are several reasons to suggest that anti-EGFR MAbs and 8-Cl-cAMP interfere with endogenous growth factor production in GEO cells. We have previously shown that 8-Cl-cAMP treat-





C

	Day 7	Day 42	Day 78	Day 119	Day 140
Control:	0.35 (±0.1)	5.4 (±0.47)	- (0)	- (0)	- (0)
8-Cl-cAMP: (0.5 mg/dose)	0.35 (±0.05)	5.27 (±0.35)	- (0)	- (0)	- (0)
mAb C225: (0.25 mg/dose)	0.35 (±0.1)	1.17 (±0.15)	3.57 (±0.6) (9)	14.2 (±0.8) (6)	15.7 (±0.9) (2)
8-Cl-cAMP + mAb C225	0.35 (±0.1)	0.23 (±0.05)	0.51 (±0.2) (10)	5.8 (±0.7) (10)	9.02 (±0.4) (7)

Fig. 3. A) Long-term effects of monoclonal antibody (MAb) C225 and/or 8-chloro-cyclic adenosine monophosphate (8-Cl-cAMP) treatment on established GEO human colon carcinoma xenografts. Experimental design is described in the legend to Fig. 2. Control (●); MAb C225, 0.25 mg/dose (○); 8-Cl-cAMP, 0.5 mg/dose (■); MAb C225, 0.25 mg/dose plus 8-Cl-cAMP, 0.5 mg/dose (□). B) Effects of MAb C225 and/or 8-Cl-cAMP treatment on the survival of GEO tumor-bearing mice. Experimental design is described in the legend to Fig. 2. a = control; b = 8-Cl-cAMP, 0.5 mg/dose; c = MAb C225, 0.25 mg/dose; and d = MAb C225, 0.25 mg/dose plus 8-Cl-cAMP, 0.5 mg/dose. Ten mice per group were monitored for survival. One of 10 mice in the MAb C225 plus 8-Cl-cAMP treatment group was tumor free and alive 30 weeks following tumor cell injection. Differences in animal survival among groups were evaluated by use of the Mantel–Cox logrank test. Mice survival was significantly different between the MAb C225-treated group and the control group; the MAb C225 plus 8-Cl-cAMP-treated group and the control group; the MAb C225 plus 8-Cl-cAMP-treated group; and the MAb C225-treated group (two-sided $P<.00001$ for each comparison). C) Tumor volume is expressed in cm^3 (± standard deviation). Numbers in parentheses represent the number of mice alive for each group. Tumor sizes among treatment groups and/or control mice were compared using the Student's t test. Differences were statistically significant as follows. Day 42: MAb C225 (0.25 mg/dose) versus control (two-sided $P<.00001$); MAb C225 (0.25 mg/dose) plus 8-Cl-cAMP (0.5 mg/dose) versus control (two-sided $P<.00001$); and MAb C225 (0.25 mg/dose) plus 8-Cl-cAMP (0.5 mg/dose) versus MAb C225 (0.25 mg/dose) ($P<.00001$). Day 78: MAb C225 (0.25 mg/dose) plus 8-Cl-cAMP (0.5 mg/dose) versus MAb C225 (0.25 mg/dose) (two-sided $P<.00001$). Day 119: MAb C225 (0.25 mg/dose) plus 8-Cl-cAMP (0.5 mg/dose) versus MAb C225 (0.25 mg/dose) (two-sided $P<.00001$). Day 140: MAb C225 (0.25 mg/dose) plus 8-Cl-cAMP (0.5 mg/dose) versus MAb C225 (0.25 mg/dose) (two-sided $P<.00001$).

ment inhibits the expression of TGF-α, at both the messenger RNA and protein levels in several transformed cell lines, and this inhibition leads to cell growth arrest (*15,16*). In this respect, 8-Cl-cAMP treatment at low doses that are not growth inhibitory determines a significant reduction of TGF-α, AR, VEGF, and bFGF protein expression in GEO tumors. Moreover, blocking EGFR activation can also interfere with TGF-α synthesis, since EGF-related growth factors enhance their expression through activation of EGFR in various cell types, including colon cancer cells (*38,39*). The suppression of synthesis of endogenous growth factors has potential therapeutic relevance. This effect could lead to tumor quiescence in terms of proliferation and neoangiogenic stimulation. Therefore, long-term treatment with anticancer agents that affect in-

tracellular signaling, such as anti-EGFR MAbs and 8-Cl-cAMP, may obtain control of cancer cell growth and spreading with n toxicity. In fact, MAb C225 and 8-Cl-cAMP treatments were well tolerated by the animals, since no signs of toxicity were observed in any treatment group.

The antitumor activity of this combination could be explored in a clinical setting. 8-Cl-cAMP can be administered at doses that are in the potential therapeutic range with no toxicity (*26*). We are currently evaluating the anticancer activity of 8-Cl-cAMP in a phase II clinical trial in patients with colorectal cancer. A phase I clinical study (*40*) has shown that MAb C225 can be given to patients with advanced cancer at doses that produce receptor-saturating levels in the blood without toxicity. Finally, since these inhibitors of intracellular mitogenic signaling have a different mechanism(s) of action and do not antagonize the effects of cytotoxic therapy, a combination of anti-EGFR MAb C225 and 8-Cl-cAMP should be investigated as a nontoxic, long-term treatment for cancer patients after chemotherapy.

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Notes

J. Mendelsohn holds stock in ImClone Systems, New York, NY, the makers of monoclonal antibody (MAb) C225; however, he does not conduct trials with MAb C225 and does not receive research support that is sponsored by ImClone Systems.

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Eradication of Established Tumors by a Fully Human Monoclonal Antibody to the Epidermal Growth Factor Receptor without Concomitant Chemotherapy

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ABSTRACT

A fully human IgG2κ monoclonal antibody (MAb), E7.6.3, specific to the human epidermal growth factor (EGF) receptor (EGFr) was generated from human antibody-producing XenoMouse strains engineered to be deficient in mouse antibody production and to contain the majority of the human antibody gene repertoire on megabase-sized fragments from the human heavy and κ light chain loci. The E7.6.3 MAb exhibits high affinity ($K_D = 5 \times 10^{-11}$ M) to the receptor, blocks completely the binding of both EGF and transforming growth factor-α (TGF-α) to various EGFr-expressing human carcinoma cell lines, and abolishes EGF-dependent cell activation, including EGFr tyrosine phosphorylation, increased extracellular acidification rate, and cell proliferation. The antibody (0.2 mg i.p. twice a week for 3 weeks) prevents completely the formation of human epidermoid carcinoma A431 xenografts in athymic mice. More importantly, the administration of E7.6.3 without concomitant chemotherapy results in complete eradication of established tumors as large as 1.2 cm^3. Tumor eradication of A431 xenografts was achieved in nearly all of the mice treated with total E7.6.3 doses as low as 3 mg, administered over the course of 3 weeks, and a total dose of 0.6 mg led to tumor elimination in 65% of the mice. No tumor recurrence was observed for more than 8 months after the last antibody injection, which further indicated complete tumor cell elimination by the antibody. The potency of E7.6.3 in eradicating well-established tumors without concomitant chemotherapy indicates its potential as a monotherapeutic agent for the treatment of multiple EGFr-expressing human solid tumors, including those for which no effective chemotherapy is available. Being a fully human antibody, E7.6.3 is expected to exhibit minimal immunogenicity and a longer half-life as compared with mouse or mouse-derivatized MAbs, thus allowing repeated antibody administration, including in immunocompetent patients. These results suggest E7.6.3 as a good candidate for assessing the full therapeutic potential of anti-EGFr antibody in the therapy of multiple patient populations with EGFr-expressing solid tumors.

INTRODUCTION

Most applications of MAbs[2] in cancer therapy rely on the ability of the antibody to specifically deliver to the cancerous tissues cytotoxic effector functions such as immune-enhancing isotypes, toxins, or drugs. An alternative approach is to use MAbs to directly affect the survival of tumor cells by depriving them of essential extracellular proliferation signals such as those mediated by growth factors through their cell receptors. One of the attractive targets in this approach is EGFr, which binds EGF and TGF-α (1–4). The binding of EGF or TGF-α to EGFr—a M_r 170,000 transmembrane cell surface glycoprotein—triggers a cascade of cellular biochemical events including EGFr autophosphorylation and internalization, which culminates in cell proliferation (1).

Several observations implicate EGFr in supporting the development and progression of human solid tumors. Overexpression of EGFr has been shown to induce transformed properties in recipient cells (5). EGFr expression has been found to be up-regulated on many human tumors including lung, colon, breast, prostate, brain, head and neck, ovarian, and renal carcinoma, and the increase in receptor levels has been reported to be associated with a poor clinical prognosis (2, 3, 6–8). In many cases, the increased surface EGFr expression was accompanied by the production of TGF-α or EGF by the tumor cells, suggesting the involvement of an autocrine growth control in the progression of these tumors (2, 3, 6, 8). These observations suggested that blocking the interaction between the growth factors and EGFr could result in the arrest of tumor growth and could possibly affect tumor survival (2–4, 6).

MAbs specific to the human EGFr, capable of neutralizing the EGF and TGF-α binding to tumor cells and of inhibiting ligand-mediated cell proliferation *in vitro*, have been generated from mice and rats (2, 4, 6). Some of these antibodies, such as the mouse 108 (9), 225, and 528 (2, 3) or the rat ICR16, ICR62, and ICR64 (6, 10, 11) MAbs, were evaluated extensively for their ability to affect tumor growth in mouse xenograft models. Most of the anti-EGFr MAbs were efficacious in preventing tumor formation in athymic mice when administered together with the human tumor cells (2, 11). When injected into mice bearing established human tumor xenografts, the mouse MAbs 225 and 528 caused partial tumor regression and required the coadministration of chemotherapeutic agents such as doxorubicin or cisplatin for the eradication of the tumors (2, 3, 12, 13). A chimeric version of the 225 MAb (C225), in which the mouse antibody variable regions are linked to human constant regions, exhibited an improved *in vivo* antitumor activity but only at high doses (14, 15). The rat ICR16, ICR62, and ICR64 antibodies caused regression of established tumors but not their complete eradication (11). These results established EGFr as a promising target for antibody therapy against EGFr-expressing solid tumors and led to human clinical trials with the C225 MAb in multiple human solid cancers (2, 3, 6). However, the limited efficacy of these MAbs as monotherapeutic agents required their assessment in combination with chemotherapy (16, 17). This requirement can limit the utilization of anti-EGFr antibodies in patients for whom chemotherapy is not available. Therefore, the identification of an anti-EGFr antibody capable of eradicating established human tumors by itself can expand the patient populations and cancer indications to which EGFr antibody therapy can be applied successfully. In addition, the MAbs currently pursued in human clinical trials, being murine chimeric antibodies (2, 4), are likely to induce immune or allergic responses to the mouse components in immunocompetent patients, which leads to reduction in the antibody efficacy and safety. Therefore, anti-EGFr antibody therapy can be fully evaluated with the availability of a fully human anti-EGFr antibody that exhibits therapeutic efficacy on EGFr-expressing tumors and that can be administered repeatedly to all appropriate patient populations.

To this end, we used our human antibody-producing XenoMouse strains to generate potent fully human anti-EGFr MAbs. As described previously (18), these mouse strains were engineered to be deficient in mouse antibody production and to contain integrated megabase-sized fragments from the human heavy and κ light chain loci with the majority of the human antibody gene repertoire. The human immunoglobulin loci provided the XenoMouse strains with the ability to produce high-affinity human MAbs to a broad spectrum of antigens including human antigens

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[2] The abbreviations used are: Mab, monoclonal antibody; EGF, epidermal growth factor; EGFr, EGF receptor; TGF, transforming growth factor.

(18, 19). As presented in this report, using XenoMouse strains we generated a panel of anti-EGFr fully human IgG2κ MAbs from which we selected the E7.6.3 antibody. This antibody exhibits high affinity ($K_D = 5 \times 10^{-11}$ M) to the receptor, neutralizes both EGF and TGF-α binding to EGFr-expressing human carcinoma cell lines, and inhibits ligand-induced tumor cell proliferation. The antibody not only prevents human tumor formation in athymic mice but, more importantly, effectively eradicates large established human tumor xenografts, independent of chemotherapeutic agents. The exhibited potent antitumor activity of E7.6.3 implies that this antibody may be applied as a monotherapeutic agent for treatment of EGFr-expressing human solid tumors.

MATERIALS AND METHODS

XenoMouse Strains. Generation and characterization of the XenoMouse-G2 strains, engineered to produce fully human IgG2κ antibodies, are described by Mendez *et al.* (18).

Human MAb Production and Purification. XenoMouse strains (8–10 weeks old) were immunized i.p with 2×10^7 A431 cells (American Type Culture Collection, CRL-7907) in complete Freunds adjuvant. Mice were boosted with the same number of A431 cells in incomplete Freunds adjuvant three times before fusion. The fusion of splenocytes from immunized mice and selection of hybridomas were carried out as described previously(18). EGFr-specific hybridomas were identified by ELISA using purified A431 cell membrane-derived EGFr (Sigma). Large quantities of antibodies were purified from ascites that were derived from severe combined immunodeficient mice inoculated with antibody-producing hybridomas, using protein-A affinity chromatography.

Measurement of Antibody Affinity to EGFr. Affinities of purified anti-EGFr antibodies were determined by plasmon resonance technology using BIAcore 2000 (Pharmacia). On the basis of the general procedures outlined by the manufacturer, kinetic analyses of the antibodies were performed using either purified membrane-derived EGFr or recombinant extracellular domain of EGFr (20) immobilized onto the sensor surface at a low density (303 RU). The association (k_{on}) and dissociation (k_{off}) rates were determined using the BIAevaluation2.1 software provided by the manufacturer. Affinity measurements of antibody in solution were carried out as described previously (18).

EGFr Binding Assays. EGFr binding assays were conducted using human recombinant ^{125}I-EGF or ^{125}I-TGF-α (Amersham Life Science, Arlington Heights, IL) as described previously(18). Briefly, human carcinoma cells growing in DMEM containing 10% FCS were detached with trypsin, washed with PBS, and resuspended in binding buffer (PBS containing 0.1% BSA (Sigma) and 0.02% NaN_3), and distributed in 96-well Multiscreen filter plates (Millipore) at 4×10^5 cells/well in 50 μl of medium. Fully human anti-EGFr or control anti-keyhole lympet hemocyanin MAbs, control human myeloma IgG2κ MAb (Calbiochem, Cambridge, MA), or mouse anti-EGFr 225 or 528 MAbs (Calbiochem) diluted in binding buffer, were added in 50 μl aliquots per well. Plates were incubated for 30 min at 4°C. ^{125}I-EGF or ^{125}I-TGF-α (0.1 nM) was added and the plates were further incubated for 90 min at 4°C. After incubation, the plates were washed five times with binding buffer, air-dried, and counted in a scintillation counter. The percentage of specifically bound ^{125}I-EGF or ^{125}I-TGF-α was calculated as follows:

$$\frac{\text{Mean cpm detected in the presence of antibody}}{\text{cpm detected in the presence of buffer only}} \times 100$$

The binding data obtained was fitted using GraphPad Prism (GraphPad Software, Inc., San Diego, CA).

***In Vitro* Tumor Cell Proliferation Assay.** The effect of antibodies on the growth of human tumor cells was determined using the method described by Ishiyama *et al.* (21). Briefly, 2×10^3 cells in 100 μl of DMEM:F12 growth medium without serum were seeded into each well of a 96-well plate. Aliquots of each diluted antibody (100 μl/well) were added in triplicate to the wells and the cultures were incubated at 37°C for 5 days. The controls consisted of either medium alone or medium containing dilutions of an human myeloma IgG2κ control antibody. After incubation, the medium was removed from each well by aspiration. All of the cells were fixed with 0.25% glutaraldehyde, then washed in 0.9% NaCl, air-dried, and stained with Crystal Violet (Fisher Scientific, Pittsburgh, PA) for 15 min at room temperature. After washing with tap water and air-drying, 100 μl of methanol was added to each well and the absorbance at 595 nm (A^{595}) of each well was determined in a Spectra Max spectrophotometer (Molecular Devices, Sunnyvale, CA). The percentage of growth inhibition is calculated as follows:

$$\frac{\text{Mean } A^{595} \text{ measured in medium only} - A^{595} \text{ in the presence of antibody}}{\text{Mean } A^{595} \text{ in the presence of medium only}} \times 100$$

EGFr Phosphorylation. Seventy % confluent A431 cells were preincubated overnight with 0.5% of fetal bovine serum at 37°C. The cells were then treated with 16 nM EGF in the absence or presence of different concentrations of E7.6.3 MAb for 30 min at 37°C. After the 30-min incubation, the cells were washed three times with cold PBS and scraped into 0.5 ml of lysis buffer (10 mM Tris, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.1 mg/ml PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 mM sodium orthovanadate). After 30 min of incubation on ice, the lysates was centrifuged at 10,000 rpm for 5 min in an Eppendorf microcentrifuge at 4°C. The protein concentration in the supernatant was measured using BCA protein assay reagents (Pierce). A small portion of the supernatant was mixed with sample buffer (Novex, San Diego, CA) and boiled for 3 min. The proteins in the supernatant were then separated by 12% SDS-PAGE. Equal amounts of total protein were loaded from each cell lysate. Mouse antiphosphotyrosine (Zymed Laboratories, South San Francisco, CA) was used for the detection of EGFr tyrosine phosphorylation on Western blots. Enhanced chemiluminescence Western blotting detection reagents (Amersham) and the Hyperfilm Enhanced chemiluminescence (Amersham) were used for visualizing the signal. The integrated densities of the bands of interest were analyzed using an AGFA Scanner and the Scanalytics OneDscan software (Hewlett Packard, Mountain View, CA).

Measurement of Cell Activation by Cytosensor Microphysiometry. To assess the effect of antibody on EGF-mediated signaling, Cytosensor Microphysiometry (Molecular Devices) was used. The Cytosensor detects early biochemical events in cell activation based on increases in the rate of acid release by the cells (22). Acid release was measured as described in the user's manual provided by Molecular Devices, Inc. Briefly, A431 cells (5×10^4) were seeded in 1 ml of medium in a Cytosensor cell capsule and cultured at 37°C for 24 h. After incubation, the cell capsules were assembled and loaded in the Cytosensor sensing chamber maintained at 37°C. The chamber was perfused with low buffer RPMI 1640 containing 1 mM sodium phosphate and 1 mg/ml endotoxin-free BSA. Acid release rates were determined with 30-s potentiometric pH measurements after an 85-s pump cycle and 5-s delay (120-s total cycle time). Basal acid release rates ranged from 60 to 120 mV/s. Percent inhibition is calculated as follows:

$$\frac{\text{Acid release in the presence of EGF only} - \text{acid release in the presence of EGF and antibody}}{\text{Acid release in the presence of EGF only}} \times 100$$

Tumor Xenograft Mouse Models. Male BALB/c-nu/nu mice (6–8 weeks of age) were injected s.c. with 5×10^6 A431 or MDA-MB-468 (American Type Culture Collection, HTB-132) cells in 100 μl of PBS. Tumor sizes were measured in a blind fashion twice a week with a vernier caliper and tumor volume was calculated as the product of length × width × height × π/6. Mice with established tumors were randomly divided into treatment groups. Animals were treated with antibodies using different regimens. Typically, mice received antibody treatment twice a week over three consecutive weeks either concomitant with the tumor cell injection (prophylactic treatment) or after tumor establishment (therapeutic treatment). The mice were followed for tumor xenograft growth and survival for at least 60 days.

Tumor Histopathological Evaluation. Biopsies obtained from athymic mice carrying human xenografts were fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned. The sections were then stained with H&E as described previously (23).

RESULTS

High-Affinity Neutralizing Fully Human Anti-EGFr MAbs from XenoMouse Strains. XenoMouse-G2 strains that produce fully human IgG2κ antibodies were immunized with the human vulvar

epidermoid carcinoma A431 cells. These cells express approximately 1×10^6 EGFr/cell (Refs. 2, 3 and data not shown). Fusion of B cells from immunized XenoMouse strains with mouse myeloma cells yielded a panel of 30 hybridomas that secrete human IgG2κ MAbs specific to the extracellular domain of human EGFr, as determined by ELISA, BIAcore, Western blots, and flow cytometry analysis (data not shown). The human γ2 was chosen as the preferred isotype with minimal immune-associated cytotoxicity against EGFr-expressing normal tissues.

To identify human MAbs with neutralization activity, purified antibodies were evaluated for their ability to block the binding of EGF and TGF-α to human tumor cell lines that express low (colon carcinoma SW948, 5×10^4/cell) or high (A431, or breast adenocarcinoma MDA-MB-468, 1×10^6/cell) levels of EGFr. As positive controls, the commercially available murine MAbs, 225 and 528, were tested in parallel. A XenoMouse-derived IgG2κ antibody PK16.3.1, specific for keyhole lympet hemocyanin, or a nonspecific human myeloma IgG2κ antibody were used as a negative control. Fig. 1*A* represents the results obtained with a subset of the fully human anti-EGFr MAbs tested in these assays. Three of the five human anti-EGFr antibodies shown, E7.6.3, E2.5.1, and E2.3.1, and the mouse anti-EGFr 225 and 528 MAbs blocked the binding of ^{125}I-EGF (0.1 nM) to A431 in a concentration-dependent manner. In contrast, E7.5.2 and E7.8.2 did not have any effect on EGF binding. The calculated IC_{50} values (3.0 nM for E7.6.3, 5.6 nM for E2.5.1, 9.1 nM for E2.3.1, 8.8 nM for 225, and 15.2 nM for 528) suggested E7.6.3 as a potent neutralizing antibody. Furthermore, EGF binding to SW948 cells was also blocked by the human E7.6.3 and E2.3.1 and by the mouse 225 MAbs (Fig. 1*B*). The IC_{50} values detected in studies with SW948 cells were 0.9 nM for E7.6.3, 0.24 nM for E2.3.1, and 0.17 nM for 225. The efficacy of E7.6.3 in neutralizing ligand binding was also demonstrated in blocking TGF-α binding to A431 cells (data not shown). These results indicated that XenoMouse strains are capable of producing fully human anti-EGFr antibodies that recognize different epitopes on the receptor, including those involved in ligand binding.

The affinity of the purified E7.6.3 MAb to EGFr was determined to be 5×10^{-11} M by both solid phase and solution measurements (K_{on}, 1.97×10^6; K_{off}, 1.13×10^{-4}). E7.6.3 exhibits cross-reactivity with African Green monkey EGFr but not with the mouse EGFr (data not shown). The E7.6.3 hybridoma exhibited significant levels of antibody production that reached a specific productivity rate of 12 pg/cell/day in serum-free medium growth conditions. On the basis of its high affinity to EGFr and its potency in blocking EGF/TGF-α binding, E7.6.3 MAb was selected for further evaluation of its efficacy in affecting tumor cell growth *in vitro* and *in vivo*.

E7.6.3 MAb Inhibits EGF-mediated Tumor Cell Activation. The ability of E7.6.3 to inhibit tumor cell activation was evaluated by examining its effects on EGF-triggered cellular responses such as the tyrosine kinase activity of EGFr, the extracellular acidification rate, and cell proliferation.

One of the first events after EGF binding to its receptor is the induction of EGFr tyrosine kinase activity, which results in the autophosphorylation of the receptor (1). As shown in Fig. 2, incubation of human EGF (16 nM) with A431 cells induced the tyrosine phosphorylation of the M_r 170,000 EGFr. While E7.6.3 did not activate the receptor tyrosine kinase activity, the antibody blocked EGF-triggered EGFr tyrosine phosphorylation in a dose-dependent manner, with a nearly complete inhibition at a concentration of 133 nM (antibody: EGF molar ratio, 8:1; Fig. 2).

Engagement of EGF with its receptors results in cell activation, which is reflected by changes in the extracellular acidification rate. These changes can be detected by the Cytosensor Microphysiometer, a pH-sensitive silicon sensor that measures real-time changes in the acidificati n of the microenvironment surrounding a population of

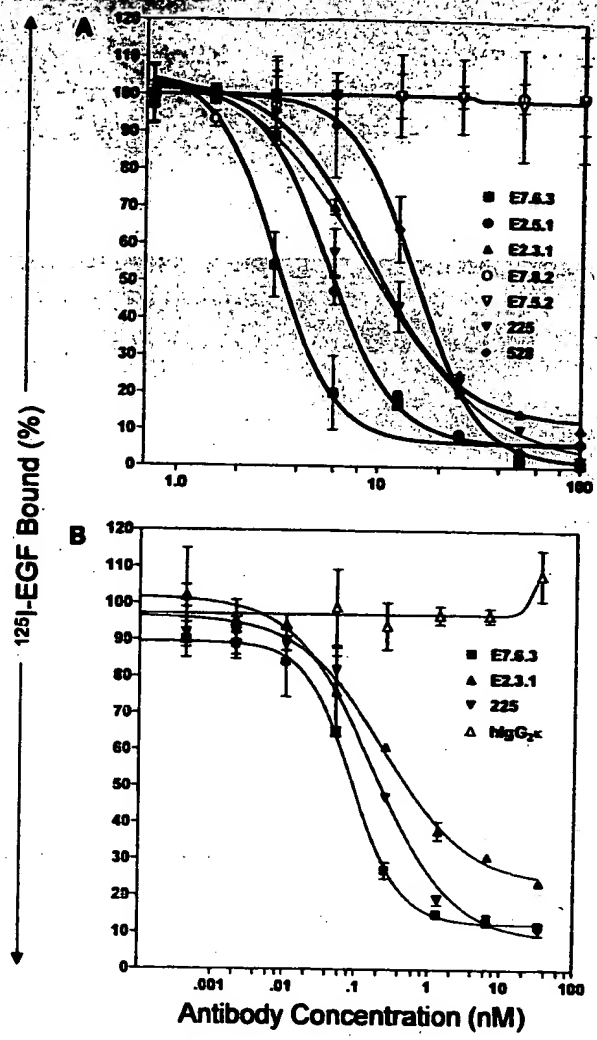

Fig. 1. The inhibition of EGF binding to EGFr by anti-EGFr MAbs. The binding of ^{125}I-EGF (0.1 nM) to (*A*) A431 or (*B*) SW948 cells was determined in the presence of human (■, E7.6.3; ●, E2.5.1; ▲, E2.3.1; ▽, E7.5.2; ○, E7.8.2) or murine (▼, 225; ◆, 528) anti-EGFr antibodies, or in the presence of the human IgG2κ control antibody (△, hIgG$_2\kappa$). The binding of ^{125}I-EGF to the cells in the absence of antibodies was designated as 100%. The data shown are representative of multiple experiments.

stimulated cells (22). Using this assay, we examined the effect of E7.6.3 on EGF-mediated A431 cell activation. As shown in Fig. 3*A*, the addition of 1.67 nM EGF to A431 cells induced an immediate increase in the extracellular acidification rate. No effect was observed when the cells were incubated only with E.7.6.3 antibody at concentrations up to 100 nM (not shown). The concurrent addition of E7.6.3 resulted in a dose-dependent inhibition of EGF-mediated extracellular acidification (Fig. 3, *A* and *B*), whereas no effect was detected with the isotype-matched control antibody PK16.3.1 (Fig. 3*B*).

Lastly, we examined the effect of E7.6.3 on the *in vitro* proliferation of the EGFr-expressing tumor cell lines A431 and MDA-MB-468, again in comparison with the mouse anti-EGFr antibodies. Both cell lines, expressing high levels of EGFr, have been shown to secrete TGF-α and to be growth-inhibited by the addition of exogenous EGF at nM concentrations (24, 25). Therefore, the experiments using these two cell lines were carried out in the absence of exogenous EGF. E7.6.3 inhibited the growth of A431 cells in a dose-dependent manner, with a maximal inhibition of 60%, a level similar to that obtained with the mouse antibody 225 and higher than that observed for the 528 antibody (Fig. 4*A*). The control antibody did not have any effect on the cell proliferation (Fig. 4*A*). The calculated IC_{50} values for E7.6.3 (0.125 nM), 225 (0.48 nM), or 528 (0.66

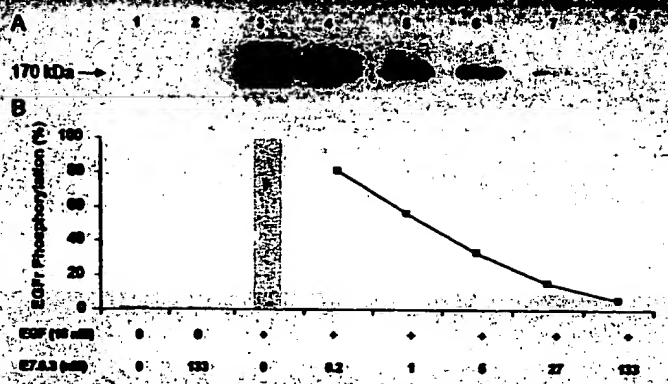

Fig. 2. The inhibition of EGF-induced tyrosine phosphorylation of EGFr by E7.6.3 MAb. A431 cells were incubated with or without 16 nM EGF, in the absence or presence of increasing concentrations of E7.6.3 MAb (0.2–133 nM) for 30 min. Cell lysates were separated by PAGE as described in "Materials and Methods." Equal amounts of total protein from the different cell lysates were loaded in each lane (not shown). EGFr tyrosine phosphorylation in cell lysates was visualized (*A*) and quantitated (*B*) using Western blot analysis and an antiphosphotyrosine antibody as described in "Materials and Methods." EGF-induced EGFr tyrosine phosphorylation in the absence of antibodies was designated as 100%.

nM) antibodies, indicated E7.6.3 efficacy in inhibiting A431 cell proliferation (Fig. 4*A*). A similar level of growth inhibition by E7.6.3 was observed with MDA-MB-468 cells (Fig. 4*B*). Because no exogenous EGF was added to the culture, these results indicate the ability of the human antibody to block autocrine growth stimulation and thus to inhibit EGF/TGF-α-induced tumor cell activation. In experiments carried out with SW948 cells, 10 nM E7.6.3 MAb blocked completely the proliferation of the cells (data not shown).

E7.6.3 MAb Prevents Human Tumor Formation in Mice. To examine the effect of E7.6.3 on tumor cell growth *in vivo*, the antibody was first evaluated for its ability to prevent the formation of A431 tumor xenografts in athymic mice. A431 cells (5×10^6/mouse) were injected s.c. into mice in conjunction with i.p. administration of PBS (group 1), 1 mg of the control antibody PK16.3.1 (group 2), or 0.2 mg or 1 mg of E7.6.3 (groups 3 and 4). The antibody administration was repeated twice a week for 3 weeks, for a total dose of 1.2 mg (group 3) or 6 mg (groups 2 and 4). As shown in Table 1, all of the mice treated with either PBS or the control antibody developed tumors by day 10 after inoculation and were killed at day 30 because of the large size of the tumors. In contrast, none of the mice treated with E7.6.3 antibody developed tumors for more than 8 months after the last antibody injection. The data indicated that E7.6.3 prevented the formation of A431 xenografts, probably by exerting its neutralization activity at the initial phase of the tumor cell proliferation.

E7.6.3 MAb Eradicates Large Human Tumor Xenografts. The effect of E7.6.3 on the growth of established tumors was examined on A431 tumor xenografts that reached a size of 0.13–1.2 cm^3 (calculated as length × width × height × $\pi/6$). Initially, mice bearing 0.13- to 0.25-cm^3-sized tumors were treated i.p. with 1 mg of either E7.6.3 Mab or the human myeloma IgG2κ control antibody, twice a week for 3 weeks. As shown in Fig. 5*A* and in Table 2, the tumors in untreated mice or in mice treated with the control antibody continued their aggressive growth to reach the size of 3 cm^3 by day 30, at which point the mice were killed. In contrast, treatment with E7.6.3 not only arrested further growth of the tumors but also caused continuous tumor regression that resulted in a complete tumor eradication in all of the mice treated (Fig. 5*A*, Table 2). No recurring tumors were detected for more than 250 days in any of the mice that were monitored, demonstrating a long-lasting effect of the antibody and its ability to completely eliminate all of the tumor cells.

We next evaluated the potency of E7.6.3 antibody to treat large established tumor xenografts. Mice bearing 0.13-, 0.56-, 0.73-, or 1.2-cm^3-sized A431 tumors were treated i.p. with 1 mg of E7.6.3 twice a week for 3 weeks, initiated on day 7, 11, 15, or 18, respectively. As demonstrated in Fig. 5*B*, E7.6.3 caused a profound tumor regression in all of the treated mice regardless of their initial tumor size, even with tumors as large as 1.2 cm^3. Furthermore, this treatment led to a complete disappearance of the tumors (Fig. 5*B*) and no tumor recurrence in any of the mouse groups for 210 days after the last antibody injection (data not shown). As summarized in Table 2, the antibody effect appears to be dose-dependent with a total dose of 3 mg leading nearly to a complete tumor eradication and a total dose of 0.6 mg eliminating 65% of the established A431 xenografts.

To compare the antitumor activity of E7.6.3 to that of the mouse 225 antibody, which was reported to affect the growth of established A431 tumors but not cause their elimination (12, 13), we used suboptimal E7.6.3 doses (0.05 mg and 0.2 mg, given twice a week for 3 weeks) that also caused primarily tumor regression in A431 xenografts. At these antibody doses, there was a significant difference between the ability of E7.6.3 and 225 to arrest the growth of A431 xenografts (Fig. 5*C*).

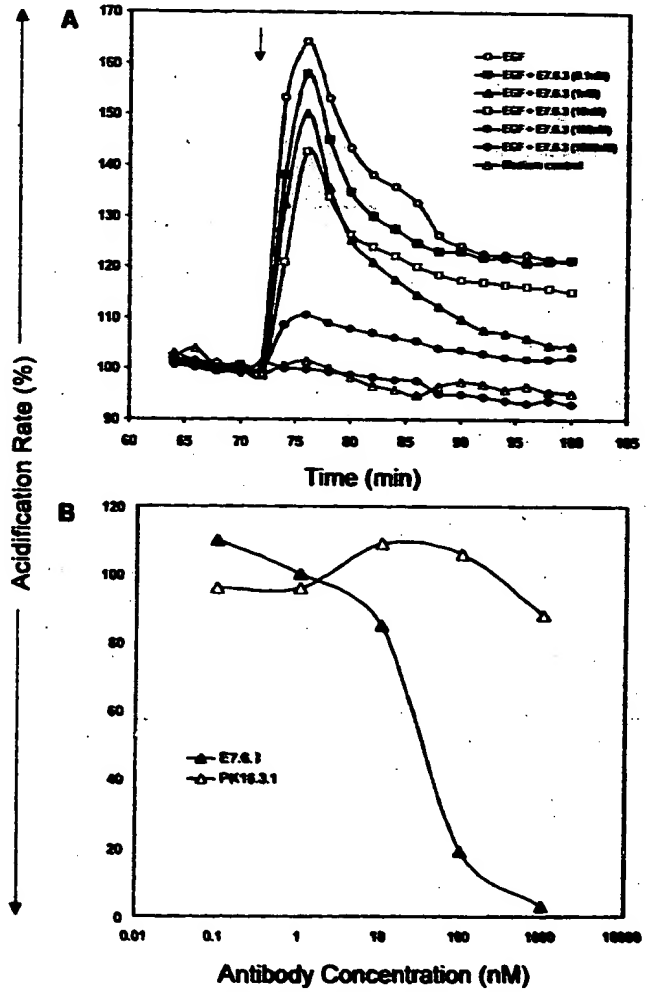

Fig. 3. The inhibition of EGF-mediated cell activation by anti-EGFr antibodies. *A*, activation of A431 cells by 1.67 nM EGF, in the absence or presence of different concentrations (0.1–1000 nM) of E7.6.3, was measured by Cytosensor as changes in extracellular acidification rate. *Arrow*, the time when EGF and/or E7.6.3 were added to the cells. The response is presented as % of baseline acidification rate (designated as 100%). *B*, the effect of increasing concentrations of E7.6.3 and control PK16.3.1 antibodies on A431 cell activation induced by 1.67 nM EGF as determined by Cytosensor. The response to EGF was measured at the peak acidification rate shown in *A*. The response in the absence of antibodies was designated as 100%. The data shown are representative of two different experiments.

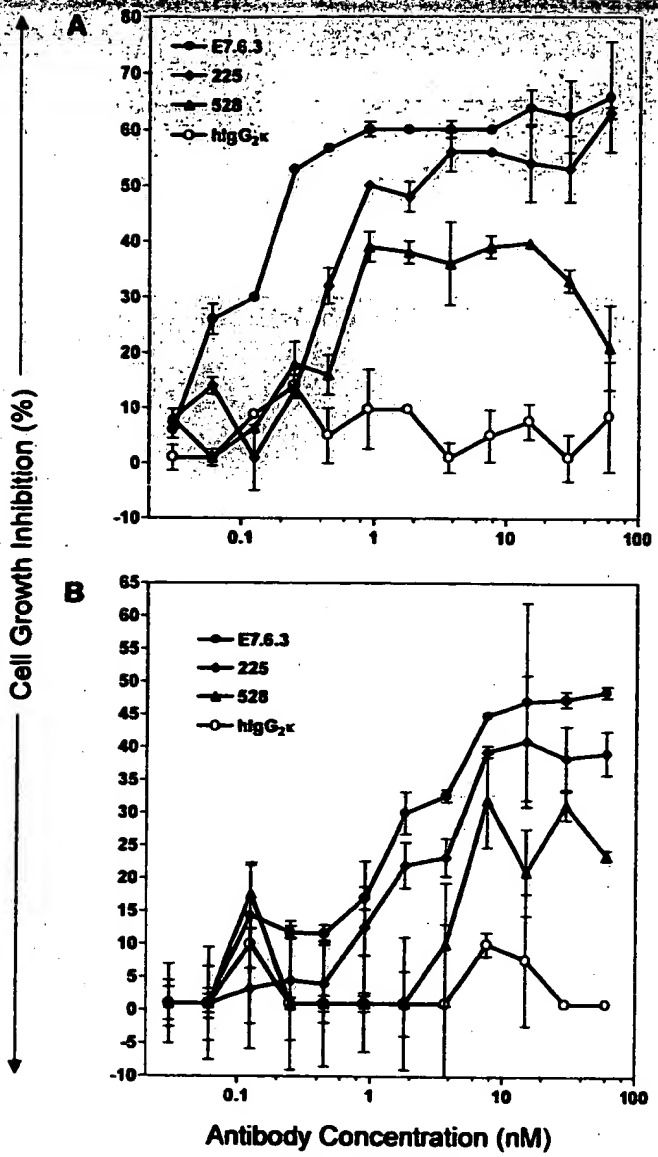

Fig. 4. The inhibition of *in vitro* tumor cell proliferation by anti-EGFr antibodies. A431 (*A*) or MDA-MB-468 (*B*) cells were cultured with anti-EGFr MAbs (●, E7.6.3; ◆, 225; ▲, 528) or control human myeloma IgG2κ antibody (○, hIgG$_2$κ), as described in "Materials and Methods." Cell viability was assayed by crystal violet staining. Data are presented as % of cell growth inhibition.

E7.6.3 was also shown to be efficacious in inhibiting the growth of the breast carcinoma MDA-MB-468 xenografts (Fig. 6*A*). Treatment of 0.2-cm^3-tumor-bearing mice with 2 mg of antibody once a week for 2 weeks led to a complete arrest of the tumor growth, with no apparent growing tumors for 140 days after the last antibody administration.

A similar anti-tumor activity of E7.6.3 was observed when the antibody was given via different administration routes (Fig. 6*B*). Administration of 0.5 mg of E7.6.3 into mice carrying 0.15-cm^3-sized A431 xenografts twice a week for three weeks by i.p., s.c., i.v., or i.m route all caused complete tumor eradication.

The elimination of all of the tumor cells by E7.6.3 was further supported by the histopathological analysis of the small residual nodules observed in some of the A431 xenograft-bearing mice that were treated with the lower antibody doses. Biopsies taken from these nodules at day 79 were shown to contain a thin fibrovascular capsule lined by necrotic cells with its center filled with keratinic and calcified debris (Fig. 7*A*). There was no evidence of neoplastic cells, which were readily detected in tumor taken from mice treated with PBS or control antibody (Fig. 7*B*). A mild inflammatory infiltration of neutrophils, lymphocytes, plasma cells, macrophages, and multinucleated giant cells surrounded the capsule. Taken together with the long-lasting inhibitory effect, the data strongly indicate complete tumor cell eradication by E7.6.3 antibody.

DISCUSSION

Utilization of XenoMouse strains for the production of human antibodies specific to the human EGFr yielded the fully human IgG2κ Mab, E7.6.3, characterized by high affinity and strong neutralization activity. Its demonstrated efficacy in eradicating large established human tumor xenografts without concomitant chemotherapy strongly suggests that it is a suitable candidate for antibody monotherapy in patients with EGFr-expressing tumors.

E7.6.3 exhibited strong efficacy in blocking the binding of EGF and TGF-α to EGFr on the surface of different human carcinoma cell lines, including those that express high levels of receptors (Fig. 1). The complete inhibition of ligand binding to the receptors on A431 and SW948 cells resulted in an abolishment of the signaling events triggered by EGF or TGF-α, including EGFr autophosphorylation, increased extracellular acidification rate, and cell proliferation. Our results indicate that E7.6.3 can block ligand-induced cell activation and that E7.6.3 does not act as an agonist to trigger cellular responses in EGFr-expressing tumors (Figs. 2 and 3).

The antitumor activity of E7.6.3 was examined in multiple xenograft mouse experiments in which the effects of various antibody doses on different sizes of tumors were established (Figs. 5 and 6). The results obtained from these studies demonstrated the unique antitumor properties of E7.6.3 MAb as compared with the other reported anti-EGFr antibodies. E7.6.3 not only arrested the growth of human tumor xenografts but also completely eradicated established tumors by itself, without the need for concomitant chemotherapy. Tumor eradication of A431 xenografts was achieved in nearly all of the mice treated with total doses as low as 3 mg administered over the course of 3 weeks, and a total dose of 0.6 mg led to tumor elimination in 65% of the mice (Fig. 5 and Fig. 6*B*, Table 2). In comparison, 8 mg of 225 and 10 mg of 528 antibodies, given over 4 and 10 weeks, respectively, had only a limited effect on A431 tumors and required the coadministration of chemotherapeutic drugs to achieve the elimination of the tumors (12, 13). A direct comparison between E7.6.3 and 225 MAbs at low doses demonstrated E7.6.3 as a more potent antibody in regressing established A431 tumors and arresting their growth (Fig. 5*C*). The chimeric C225 MAb, which was reported to acquire higher affinity to EGFr and enhanced *in vivo* antitumor activities, achieved complete A431 tumor eradication at a total dose of 10 mg given for 5 weeks, whereas total doses of 2.5 and 5 mg led to only 14% and 57% of tumor-free mice (14). The potent antitumor activity of E7.6.3 was

Table 1 *Prevention of tumor formation by E7.6.3 MAb*

On day 0, mice were injected s.c. with 5×10^6 A431 cells and i.p. with PBS, 1 mg of control antibody PK16.3.1, or 0.2 mg or 1 mg of E7.6.3 MAb twice a week for 3 weeks. Incidence of tumor formation is expressed as the number of mice with visible tumors/total number of mice within each group.

	Incidence of tumor formation			
Time (day)	PBS	PK16.3.1 (1 mg)	E7.6.3 (0.2 mg)	E7.6.3 (1 mg)
0	0/5	0/5	0/10	0/10
3	4/5	0/5	0/10	0/10
8	4/5	3/5	0/10	0/10
10	5/5	5/5	0/10	0/10
25	5/5	5/5	0/10	0/10
100	ND[a]	ND	0/10	0/10
250	ND	ND	0/10	0/10

[a] ND, not determined.

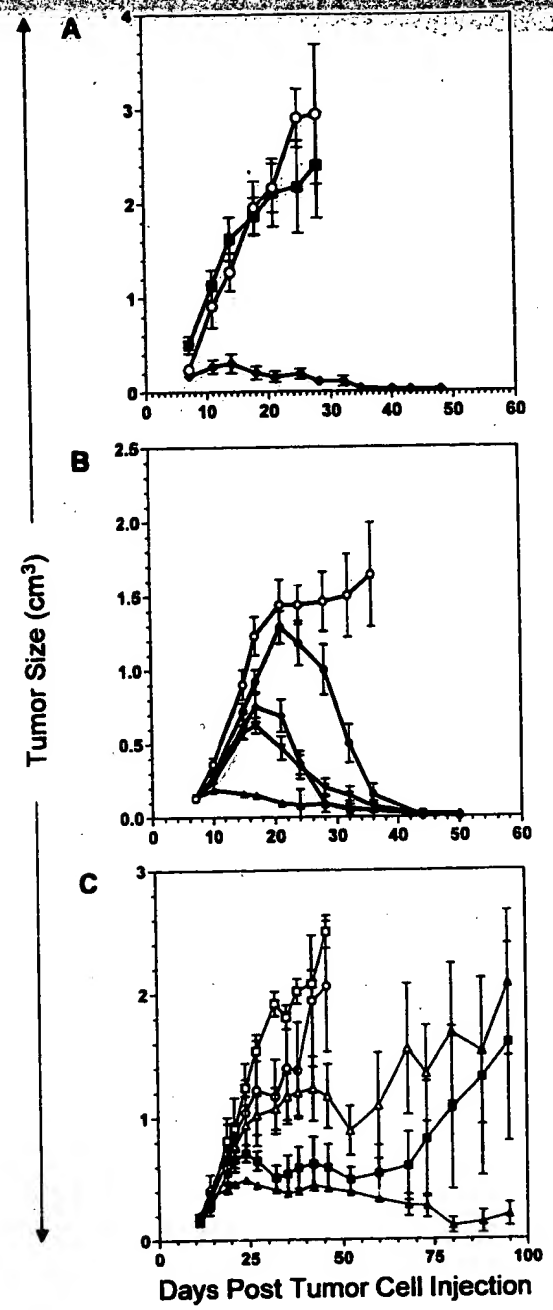

Fig. 5. The eradication of established A431 tumor xenografts by E7.6.3 MAb. A431 (5×10^6) cells were injected s.c. into nude mice on day 0. *A*, at day 7, when tumor size reached an average volume of 0.13–0.25 cm^2, mice were randomly divided into treatment groups ($n = 5$) and were injected i.p. with PBS (○) or with 1 mg of either E7.6.3 (◆) or the control human myeloma IgG2κ (■) antibody twice a week for 3 weeks. *B*, when the mean tumor sizes reached 0.13 (▲), 0.56 (▼), 0.73 (◆), or 1.2 (●) cm^3, mice ($n = 10$) were treated with 1 mg E7.6.3 twice a week for 3 weeks. Control mice (○, $n = 10$) received no treatment. *C*, at day 10, when tumor sizes reached 0.15 cm^3, mice ($n = 8$) were injected i.p. with 200 μg (▲) or 50 μg (■) doses of E7.6.3, or 200 μg (△) or 50 μg (□) doses of 225 MAbs, twice a week for 3 weeks. Control mice (○) received no treatment. Tumor volumes were measured twice a week as described in "Materials and Methods." The data are presented as the mean tumor size ± SE.

Table 2 *The effect of E7.6.3 MAb on established A431 xenograft tumors*

Nude mice with established A431 xenografts (tumor size of 0.13–0.25 cm^3 at day 7–10) were treated i.p. with various doses of E7.6.3 MAb or human myeloma $IgG_2\kappa$ control antibody twice a week for 3 weeks. The Table summarizes the results of 11 experiments. Mice that received no treatment or control $IgG_2\kappa$ antibody were sacrificed between days 35 and 50.

Treatment (dose/injection)	Total dose (mg)	Total no. of mice	Tumor-free mice on day 60	
			No.	%
None		71	0	0
Control $IgG_2\kappa$ (1 mg)	6	16	0	0
E7.6.3 (1 mg)	6	50	50	100
E7.6.3 (0.5 mg)	3	20	19	95
E7.6.3 (0.25 mg)	1.5	5	3	60
E7.6.3 (0.2 mg)	1.2	19	5	26
E7.6.3 (0.1 mg)	0.6	20	13	65
E7.6.3 (0.05 mg)	0.3	15	1	7

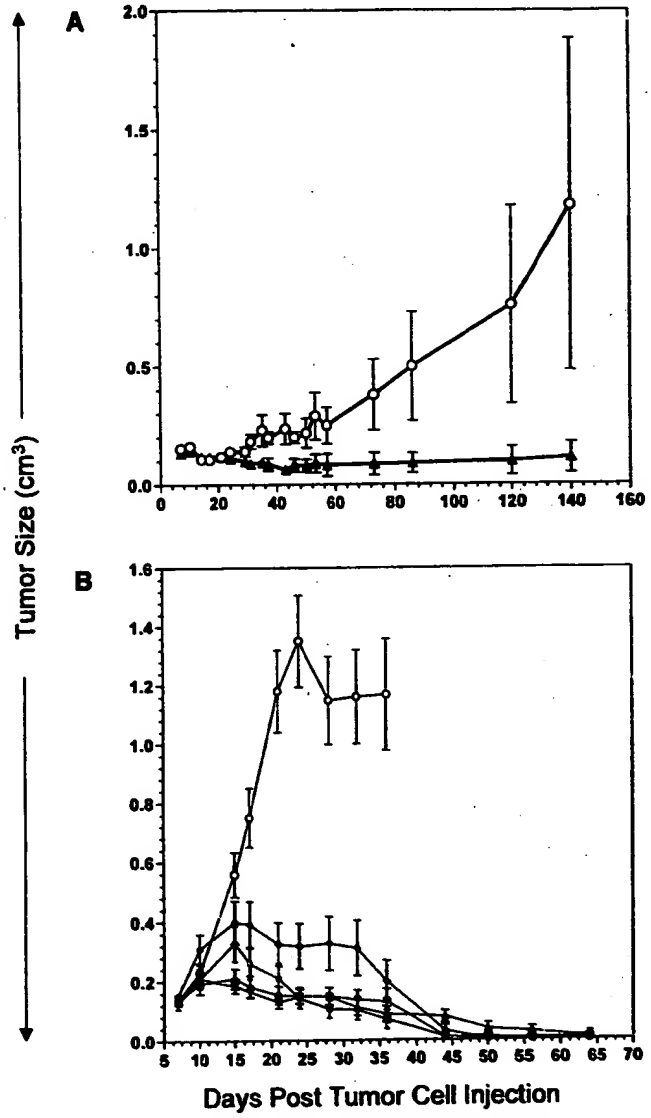

Fig. 6. The effect of E7.6.3 MAb on the growth of established human tumor xenografts. Five × 10^6 MDA-MB-468 (*A*) or A431 (*B*) cells were injected s.c. into the nude mice on day 0. *A*, 7 days after injection of MDA-MB-468 cells, mice ($n = 8$) were injected i.p. with 2 mg of E7.6.3 MAb once a week for 2 weeks (▲). Control mice ($n = 8$) received no treatment (○). *B*, mice ($n = 10$) were given 0.5 mg E7.6.3 via i.p. (■), i.v. (▲), s.c. (▼), or i.m. (◆) injections twice a week for 3 weeks. Control mice (○) received no treatment. The data represent the mean tumor size ± SE.

further validated by its ability at a 6 mg total dose to completely eliminate established tumors as large as 1.2 cm^3 in all of the mice treated.

This antitumor potency of E7.6.3 is likely to originate primarily from the intrinsic activity of the antibody inasmuch as its human γ2 isotype was shown to minimally engage the immune system-derived effector functions such as cell-mediated cytotoxicity or complement-dependent cytolysis. In comparison, the antitumor activities of the rat ICR62, mouse

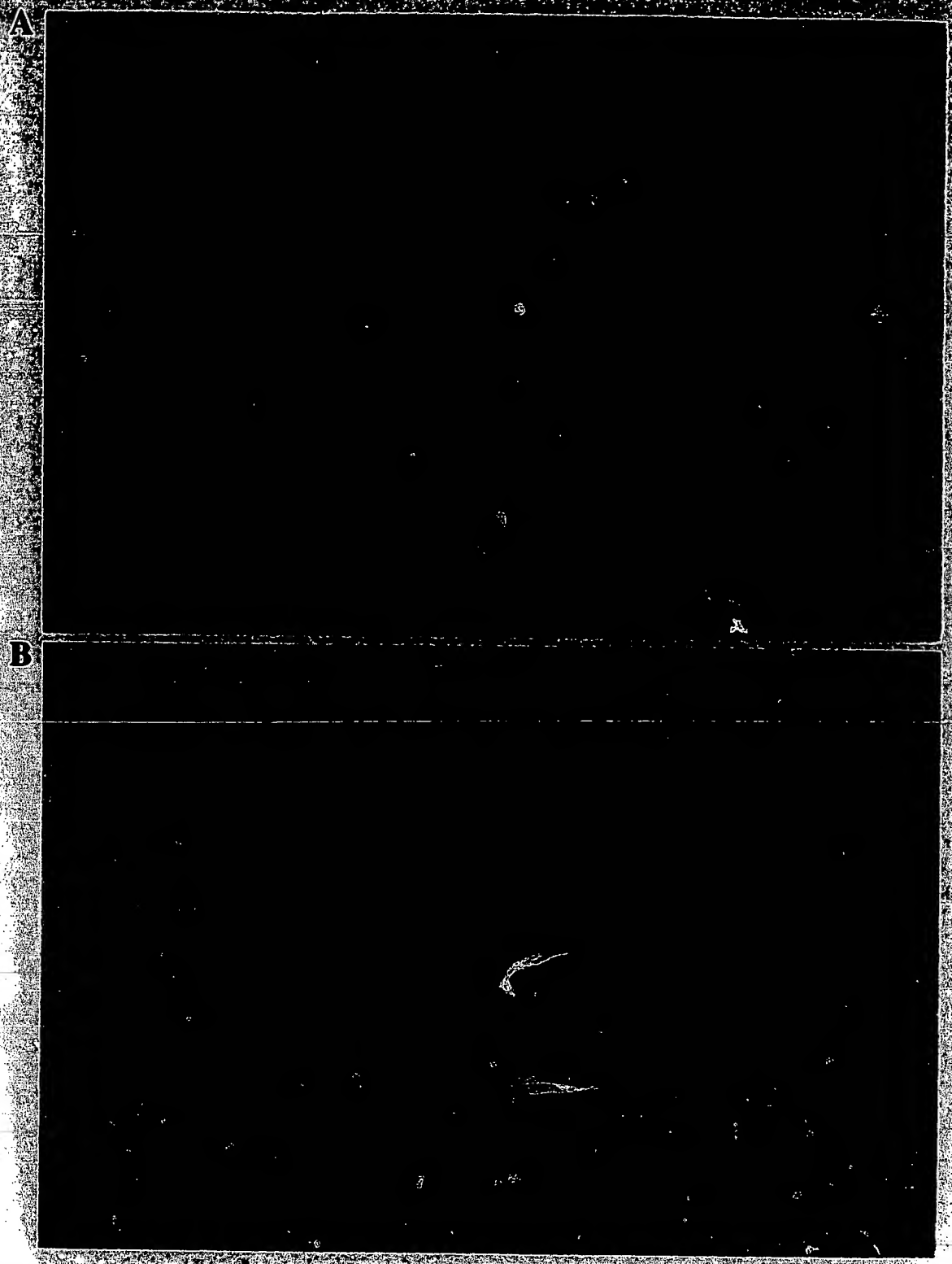

Fig. 7. Histopathology of E7.6.3-treated A431 xenografts. A, mice with established A431 xenografts were treated i.p. with 0.5 mg of E7.6.3 MAb twice a week for 3 weeks. On day 76 after tumor cell (5×10^6) injection, tumor-like nodules were excised and examined histologically as described in "Materials and Methods." *B*, histological analysis of A431 xenografts excised from an untreated mouse.

528, or chimeric C225 antibodies were suggested to reflect the participation of the host immune effector functions recruited by the respective rodent γ2b or human γ1 isotypes (2, 4, 6, 26).

The molecular mechanism(s) underlying the potent antitumor activity of E7.6.3 still remains elusive. Some hypotheses that can be proposed are based on the ability of the antibody to block ligand-triggered growth and survival signals, whereas others emphasize the possible effects that the antibody may exert upon the cell on its interaction with the receptor. The potency of E7.6.3 can be attributed, at least in part, to the high binding affinity ($K_D = 5 \times 10^{-11}$ M) to human EGFr, higher than the affinity reported for other anti-EGFr MAbs (12, 14). With its high affinity, E7.6.3 can inhibit or dissociate the ligand binding to the receptors very effec-

tively, thus depriving the cells completely from receiving essential growth and survival stimuli. Like other anti-EGFr antibodies (2, 4, 6), E7.6.3 MAb does not act as an agonist and does not activate cells on binding to the receptor. The difference in efficacy between E7.6.3 and the other antibodies tested in xenograft mouse models can also be attributed to a unique E7.6.3 binding epitope on EGFr that can mediate a stronger neutralization effect or induce cytotoxicity. The latter hypothesis is supported by the ability of E7.6.3 to eradicate well-established human xenografts as large as 1.2 cm^3. The mechanism behind the *in vivo* cytocidal effects of E7.6.3 is not yet clear and may involve the induction of programmed cell death, differentiation of the tumor cells, or modulation of expression of angiogenesis factors—mechanisms that were shown to be triggered by antibodies in cultured cells (27–31). Different mechanisms may account for the antibody effect on different tumors; and in some cases, probably more than one mechanism contributes to the antitumor activity.

The potency of E7.6.3 in eradicating well-established tumors indicates that this antibody can provide effective therapy to tumors that require EGFr activation for their continuous progression and survival. Because E7.6.3 does not require the presence of chemotherapy to exert antitumor activity, the antibody could be applied to various EGFr-expressing human solid tumors. Furthermore, being a fully human antibody, E7.6.3 is expected to have a long half-life and minimal immunogenicity with repeated administration, including in all immunocompetent patients. In addition, bearing a human γ2 constant region that interacts poorly with the effector arm of the immune system, E7.6.3 MAb may not induce cytotoxic effects on EGFr-expressing normal tissues such as liver and skin.

Utilization of Mabs directed to growth factor receptors as cancer therapeutics has been validated recently by the tumor responses obtained from clinical trials with Herceptin, the humanized anti-HER2 antibody, in patients with HER2-overexpressing metastatic breast cancer (32, 33). The potent *in vivo* antitumor activity of E7.6.3, as demonstrated in this report, suggests that it is a good candidate for assessing the therapeutic potential of anti-EGFr therapy in EGFr-expressing human tumors.

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